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# THE JOURNAL OF HYGIENE

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THE DIFFERENTIATION OF THE MANNITE-FERMENTING GROUP OF *B. DYSENTERIAE* WITH SPECIAL REFERENCE TO STRAINS ISOLATED FROM VARIOUS SOURCES IN THIS COUNTRY.

By H. DE R. MORGAN, M.A., M.R.C.S., L.R.C.P., D.P.H.

(*From the Lister Institute of Preventive Medicine, London.*)

THE present paper contains the results of an enquiry into the differentiation of certain organisms of varying source, which present characters more or less closely allied to those of the "mannite" or "Flexner" type of the dysentery bacillus.

The "non-mannite" type, first discovered by Shiga in 1898, as the cause of certain forms of bacillary dysentery in Japan, and later by Kruse in 1901, as the cause of epidemic dysentery in Rhenish Westphalia, has been shown to remain remarkably true to type, and our present cultural and serological methods amply suffice to separate it off clearly from the "mannite" or "Flexner" type. We are, in fact, justified in speaking of the Shiga bacillus of Dysentery. It is otherwise with the mannite-fermenting type. The work of the last ten years in various countries has elicited the fact that the bacillus discovered by Flexner in Manila in 1901 must be regarded merely as a type of a group comprising an ever-increasing number of strains differing in certain properties from the type-strain, and from each other.

The members, or sub-types, of the mannite-fermenting group, which have been most thoroughly investigated are Flexner's bacillus, the "Y" bacillus of Hiss and Russell discovered in 1904, and the bacillus of Strong.

I do not propose at this point to enter in detail into the differential characters of these three sub-types. It will be sufficient at present to state that the Flexner bacillus is far more closely allied on biological and serological grounds to the "Y" bacillus of Hiss and Russell than either of them is to the bacillus of Strong.

In the differentiation of the Flexner from the "Y" bacillus, Lentz (1909) lays some stress on the non-fermentation of maltose by the latter and the less marked formation of indol, but it is questionable whether this distinction is invariable (*vide infra*). Differentiation of these two bacilli by agglutination methods is only partially possible and it is only by the method of absorption that a tolerably clear distinction can be drawn between them.

The bacillus of Strong, which does not appear to have been encountered with any certainty since Strong's discovery of it in 1900, remains in a distinctly separate category not only by its ability to ferment cane sugar in addition to maltose and mannite, but also by its agglutination characteristics.

All these bacilli have been isolated from cases of dysentery in different countries, and in spite of the fact that certain differences have been found to exist between them, and that in not a few cases the bacilli of the mannite and non-mannite types have been found side by side in the same case of dysentery, there is at present no sufficient evidence in support of the suggestion that the Shiga or non-mannite type is the only sufficient cause of bacillary dysentery, and that the mannite-fermenting bacilli are merely associated organisms and not of prime aetiological importance. The prevalent notion also that the Shiga bacillus gives rise to a much severer type of dysentery than that associated with the mannite-fermenting bacilli, is probably unjustified. Very severe and fatal cases of dysentery have been found to be associated with the mannite-fermenting group also.

In this country, apart from asylums and similar institutions, bacillary dysentery has been rarely met with, and still more rarely established as such by bacteriological methods. In asylum dysentery the work of Eyre (1904) showed the presence of bacilli identical with the Shiga type. Some years previously Kruse had isolated from asylum cases his so-called pseudo-dysentery bacillus, which has since been identified more or less completely with the *Bacillus "Y"*.

McWweeney (1905) also isolated from a case of asylum dysentery a bacillus probably identical with *B. pseudo-dysenteriae* of Kruse.

Aveline, Boycott and Macdonald (1908) in their investigation in

a particular asylum, found the mannite type of bacillus, which they identified, on fermentation and agglutination grounds only, with the bacillus of Flexner.

Macalister (1910) has also recently found the mannite type in a number of asylum cases.

The only sporadic case in this country definitely associated with the dysentery bacillus is that recorded by Marshall (1909). Marshall's case was that of a child two years old who died in the month of January 1909 in Lambeth, of a very acute form of dysentery lasting barely 48 hours, accompanied by the presence of blood and mucus in the stools. The organism isolated in practically pure culture from this case, was identified by cultural and agglutination methods with the bacillus of Flexner, but the method of absorption served to distinguish it therefrom.

The American type of infantile diarrhoea is ascribed by many observers to either the Flexner or the Shiga type of *B. dysenteriae*. Duval and Bassett (1904) came to this conclusion in 1902 and their opinion was afterwards confirmed by many observers at the Rockefeller Institute in 1903. During the summer of that year an investigation was made, under the direction of Flexner, into the occurrence of the dysentery bacillus in the epidemic diarrhoea of children, the result of which was to prove the *B. dysenteriae* to be the cause of certain epidemics of summer diarrhoea in America.

That the dysentery bacillus is the cause of summer diarrhoea in England is unlikely, since, as far as I can ascertain, no bacillus exactly corresponding to the true Shiga or Flexner type has ever been isolated in this country from cases of that disease. The dysentery bacillus was searched for by Scholberg at Cardiff in cases of summer diarrhoea during the summers of 1904 and 1905, and by myself and others at the Lister Institute during the summers of 1905-6-7-8 (Morgan 1906, 1907, Morgan and Ledingham 1909), but no bacilli corresponding exactly to any known type of dysentery bacilli were ever isolated. At the same time, in each of these years, in a small number of cases, bacilli of an atypical Flexner type were found. The percentage of these found in each year was too low to admit of their being considered a possible cause of the disease, so that we came to the conclusion that these atypical bacilli were either members of the normal flora of the intestine, or were accidental in occurrence.

O. Mayer (1910) has also recently described a strain "Furth" which he isolated from an epidemic of dysentery in a German regiment. He

comes to the conclusion that although it culturally resembles *B. pseudodysenteriae* D of Kruse, it is distinguished from it by agglutination and absorption experiments. He therefore regards it as a new strain of the dysentery bacillus.

A similar strain has also been recorded by Lösener (1909).

Marshall's strain above referred to and which has also been investigated by me (see later B 1) may also require to be placed in a group by itself in virtue of the serological relations obtained with it.

Ruffer and Willmore (1909) have found a large variety of mannite-fermenting strains which they isolated from cases of dysentery in Arabia. None of these bacilli are found to be identical with any known members of this group on the application of the absorption test, and they have therefore been regarded by their discoverers as new types of the mannite-fermenting group (El Tor group).

From the foregoing examples it will be seen that recent research appears to show that undoubted cases of dysentery may be associated with bacilli more or less akin to the Flexner type but separated from it and other known sub-types by certain differential characteristics. It seemed therefore of great importance to ascertain whether, by a more extensive series of differential tests, it might be possible to group these various bacilli in a more satisfactory way.

A number of dysentery-like strains, isolated chiefly from the excreta of typhoid convalescents, or suspected typhoid "carriers," by Dr Ledingham, were placed at my disposal, so that some comparison might be made between them and known strains isolated from actual cases of dysentery in other countries. A few strains recovered from cases of dysentery at Claybury Asylum by Dr Candler were also thoroughly examined.

*Source of foreign strains. Group A (vide Table I).*

The collection of foreign strains included two strains of the bacillus of Flexner, one of Flexner-Gray, two cultures of Duval isolated from cases of infant's diarrhoea in Baltimore and New York respectively, a culture of *Bacillus "Y"* of Hiss and Russell kindly sent to me by Professor Lentz, a culture of *Bacillus Strong* from Professor Kruse, two cultures of *B. pseudodysenteriae* D of Kruse and one of his *B. pseudodysenteriae* A. To these were added thirteen strains kindly sent to me by Dr Willmore which had been isolated by Ruffer and himself from cases of dysentery occurring amongst the Arab pilgrims to Mecca at

El Tor in Arabia. Those designated by him as "El Tor" bacilli he considers to be the cause of the disease in view of their serum reactions. Willmore also sent me three strains of bacilli isolated from infant's diarrhoea in Alexandria.

*Source of British strains. Group B (vide Table II).*

- B 1. From faeces of fatal case of dysentery. Marshall's case (see above).
- B 2. From faeces of Mrs W. a suspected typhoid-carrier. No history of dysentery obtainable. Strain isolated 19 October, 1909. A second examination on 1 December, 1909 yielded no dysentery-like bacilli.
- B 3. From faeces of typhoid convalescent F. W. discharged from hospital on 22 February, 1909. Isolated 30 August, 1909. Four examinations previous to this date and one later gave no dysentery-like bacilli. *Said to have had dysentery four years ago.*
- B 4. From faeces of Miss W. daughter of Mrs W. (B 2) suspected typhoid-carrier. Isolated 1 December, 1909. No history of dysentery obtainable.
- B 5, B 6. Faeces and urine of Miss N. suspected typhoid-carrier. Isolated 21 December, 1909. Mother died three weeks previously after a confinement, with symptoms of profuse diarrhoea which had lasted a considerable time and which may possibly have been due to bacillary dysentery. There was no autopsy.
- B 7. From faeces of a child suffering from severe diarrhoea.
- B 8. From urine of Miss J. W. sister of F. W. (B 3), typhoid convalescent. Discharged from hospital 31 March, 1909. Isolated 30 August, 1909. Four examinations previous to this date and one later gave no dysentery-like bacilli. No history of dysentery.
- B 9. From faeces of Miss P. suspected typhoid-carrier. Isolated 24 September, 1909. No history of dysentery.
- B 10. From spleen of a mouse which had been fed on material suspected to have caused severe intestinal disturbance in man.
- B 11. From stool containing mucus and blood from mild case of clinical asylum dysentery. (Claybury.)
- B 12. Same source as B 11.
- B 13. Same source as B 11.
- B 14. From faeces of Miss J. W. (B 8). Isolated 7 October, 1909.
- B 15. Isolated post-mortem from scraping of intestines in a case of dysentery. (Claybury.)
- B 16. From stool containing blood and mucus. At post-mortem there were ulcers of small intestine with dysenteric ulceration of the lowest part of the large intestine. Patient had pulmonary tuberculosis. (Claybury.)
- B 17. From faeces of suspected female typhoid-carrier in an asylum. Isolated November, 1907. This strain is probably a non-motile *B. typhosus* (*vide infra*).
- B 18. From urine of Miss B. (age 7 years) typhoid convalescent. Isolated 7 February, 1910.
- B 19. From urine of S. typhoid convalescent.
- B 20. From faeces of I. P. typhoid convalescent. Isolated 17 August, 1909.

*B. dysenteriae*

- B 21. From an apparently wholesome "Cambridge" sausage.
- B 22. From faeces of F. H. suspected typhoid-carrier. Isolated 16 October, 1908.
- B 23. From urine of Miss S. (5 years) suspected typhoid-carrier. Isolated 19 October, 1909.
- B 24. From urine of Miss D. Y. suspected typhoid-carrier. Isolated 25 September, 1909.
- B 25. From urine of Miss M. Y. sister of above, suspected typhoid-carrier. Isolated 28 September, 1909.

With regard to the strains B 2, B 3, B 4, B 5, B 6, B 8, B 9, B 14, B 17, B 18, B 19, B 20, B 23, B 24 and B 25 isolated by Dr Ledingham from typhoid convalescents or healthy persons examined in the course of searches in various districts for typhoid-carriers, it has to be admitted that definite evidence of a past or present attack of dysentery was in the majority of cases not forthcoming. There is little doubt, however, in view of the results presently to be detailed, that some at least of these strains must be regarded as genuine examples of the mannite-fermenting dysentery-group and that the harbourers of such strains may very possibly have suffered from some mild form of diarrhoea whose dysenteric origin was unrecognised.

In order to make an exhaustive comparison of these cultures, each of them was (1) tested by its cultural reactions when grown on various media, (2) by agglutination experiments with known sera, and (3) by absorption experiments with the same sera, besides which the virulence of some of them was tested on animals.

*Cultural reactions.*

To test their cultural reactions these bacilli were grown on solutions in peptone water of all the sugars, alcohols and glucosides obtainable, also on litmus-milk, peptone-beef-broth and gelatine. Before determining these reactions all the cultures had been plated and a separate colony picked off to ensure purity.

The gelatine cultures were grown at room temperature, liquefaction being looked for at the end of two months. The growth on the various sugars &c. took place at 37° C. and their reactions were noted at the end of two, four and seven days.

The litmus-milk tubes were examined at the end of one, three and fifteen days whilst motility was determined in broth cultures grown for five hours. Indol was tested for at the end of five days' growth on broth.

TABLE I. (*Foreign Strains.*)

Designation	Lactose										Galactose									
	N	S	K	X	Z	Y	W	V	U	T	R	P	Q	S	N	M	L	O	M	P
<i>B. Pfeiffer "L. L. P. M."</i> ...	..	A.1	NMB	a	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>B. Pfeiffer "Elstree"</i> ...	..	A.2	NMB	a	a	-	-	-	b	alks	alks	+	+	-	-	-	-	-	-	-
<i>B. Pfeiffer Gray</i> ...	..	A.3	NMB	a	a	-	-	-	a	alks	alks	+	-	-	-	-	-	-	-	-
<i>B. Duerl's diarrhoea</i> Baltimore A.4	..	A.4	NMB	a	a	-	-	-	a	alks	alks	+	-	-	-	-	-	-	-	-
<i>B. Duerl's diarrhoea</i> New York A.5	..	A.5	NMB	a	a	-	-	-	b	alks	alks	+	-	-	-	-	-	-	-	-
<i>B. "Y" Hiss and Russell (Lentz)</i> ...	..	A.6	NMB	a	a	-	-	-	as	alks	alks	+	-	-	-	-	-	-	-	-
<i>B. Strong (Kruse)</i> ...	..	A.7	NMB	a	a	-	-	-	b	alks	alks	+	-	-	-	-	-	-	-	-
<i>B. pseudo-dysenteriae</i> A. Kruse	..	A.8	NMB	a	a	-	-	-	as	A	++	-	-	-	-	-	-	-	-	-
<i>B. pseudo-dysenteriae</i> D. Kruse	..	A.9	NMB	a	a	-	-	-	as	alks	++	-	-	-	-	-	-	-	-	-
<i>B. pseudo-dysenteriae</i> D. Kruse	..	A.10	NMB	a	a	-	-	-	as	alks	++	-	-	-	-	-	-	-	-	-
<i>B. Pfeiffer</i> Willmore (El Tor)	..	A.11	NMB	a	a	-	-	-	as	alks	++	-	-	-	-	-	-	-	-	-
<i>B. pseudo-dysenteriae</i> Willmore	..	A.12	NMB	a	a	-	-	-	as	o	alks	++	-	-	-	-	-	-	-	-
<i>B. pseudo-dysenteriae</i> Willmore	..	A.13	NMB	a	a	-	-	-	as	alks	++	a	-	-	-	-	-	-	-	-
<i>B. pseudo-dysenteriae</i> Willmore	..	A.14	NMB	a	a	-	-	-	as	o	alks	++	a	-	-	-	-	-	-	-
<i>B. Tor 22</i> Willmore ...	..	A.15	NMB	a	a	-	-	-	as	alk	++	a	-	-	-	-	-	-	-	-
<i>B. Tor R.H.B.</i> Willmore ...	..	A.16	NMB	a	a	-	-	-	a	alks	++	-	-	-	-	-	-	-	-	-
<i>B. Tor R.E.T.</i> Willmore ...	..	A.17	NMB	a	a	-	-	-	as	alks	++	-	-	-	-	-	-	-	-	-
<i>B. Tor 44</i> Willmore ...	..	A.18	NMB	a	a	-	-	-	as	alks	++	-	-	-	-	-	-	-	-	-
<i>B. Tor 12/10</i> Willmore ...	..	A.19	NMB	a	a	-	-	-	as	alks	-	-	-	-	-	-	-	-	-	-
<i>B. Tor 26/7</i> Willmore ...	..	A.20	NMB	a	a	-	-	-	as	alks	-	-	-	-	-	-	-	-	-	-
<i>B. Tor 167</i> Willmore ...	..	A.21	NMB	a	a	-	-	-	as	alks	++	a	-	-	-	-	-	-	-	-
<i>B. infant's diarrhoea</i> Willmore ...	..	A.22	NMB	a	a	-	-	-	as	alks	++	-	a	-	-	-	-	-	-	-
<i>B. infant's diarrhoea</i> Willmore ...	..	A.23	NMB	a	a	-	-	-	as	alks	++	-	a	-	-	-	-	-	-	-
<i>B. infant's diarrhoea</i> Willmore ...	..	A.24	NMB	a	a	-	-	-	as	alks	++	-	a	-	-	-	-	-	-	-
<i>B. 162 U.P.</i> Willmore ...	..	A.25	NMB	a	a	-	-	-	a	alks	++	-	-	-	-	-	-	-	-	-
<i>B. 165 U.P.</i> Willmore ...	..	A.26	NMB	a	a	-	-	-	as	alks	++	a	-	-	-	-	-	-	-	-

N.R.—NMB = Non-motile bacillus.

a = acid,  
b = slight acid.  
as = neutral.

7 = as = 7th day slightly acid.

4 = as = 4th day slightly acid.

alks = slightly alkaline.

4 = as = 4th day alkaline.

7 = as = 7th day alkaline.

### *B. dysenteriae*

TABLE II. (*British strains.*)

Lactus milk		Group I		Group II		Group III		Group IV	
Age	Days	Age	Days	Age	Days	Age	Days	Age	Days
B 1 NMB	0	A 0	0	+ +		A 0	-	-	-
B 2 NMB	0	A 0	0	+ +		A 0	-	-	-
B 3 NMB	0	A 0	0	+ +		A 0	-	-	-
B 4 NMB	0	A 0	0	+ +		A 0	-	-	-
B 5 NMB	0	A 0	0	+ +		A 0	-	-	-
B 6 NMB	0	A 0	0	+ +		A 0	-	-	-
B 7 NMB	0	A 0	0	+ +		A 0	-	-	-
B 8 NMB	0	A 0	0	+ +		A 0	-	-	-
B 9 NMB	0	A 0	0	+ +		A 0	-	-	-
B 10 NMB	0	A 0	0	+ +		A 0	-	-	-
B 11 NMB	0	A 0	0	+ +		A 0	-	-	-
B 12 NMB	0	A 0	0	+ +		A 0	-	-	-
B 13 NMB	0	A 0	0	+ +		A 0	-	-	-
B 14 NMB	0	A 0	0	+ +		A 0	-	-	-
B 15 NMB	0	A 0	0	+ +		A 0	-	-	-
B 16 NMB	0	A 0	0	+ +		A 0	-	-	-
B 17 NMB	0	A 0	0	+ +		A 0	-	-	-
B 18 NMB	0	A 0	0	+ +		A 0	-	-	-
B 19 NMB	0	A 0	0	+ +		A 0	-	-	-
B 20 NMB	0	A 0	0	+ +		A 0	-	-	-
B 21 MB	0	A 0	0	+ +		A 0	-	-	-
B 22 MB	0	A 0	0	+ +		A 0	-	-	-
B 23 MB	0	A 0	0	+ +		A 0	-	-	-
B 24 MB	0	A 0	0	+ +		A 0	-	-	-
B 25 MB	0	A 0	0	+ +		A 0	-	-	-

The results of these cultural reactions will be seen in Tables I and II.

From Table I it will be noticed that the first six cultures isolated from epidemics of dysentery and from cases of dysenteric diarrhoea in America give exactly the same reactions on all the media used, whereas the reactions of none of the others are exactly alike, nor do any of them conform in every particular to the reactions given by the members of the Flexner group. The bacillus of Strong ferments dulcite, cane sugar and sorbite, and also clots milk, thus differing from all the bacilli under investigation.

The strain of *Bacillus "Y"* received from Professor Lentz was found to ferment maltose and thus correspond completely in its fermentation characters with the Flexner strains. It is possible, however, that this strain may have had some previous training on maltose and Hiss (1904) had already shown that his bacillus though unable to ferment maltose when isolated from the body may readily acquire that power by a process of training on a medium containing the sugar. The great majority of the so-called pseudo-dysentery strains of Kruse including those isolated by Willmore at El Tor do not ferment maltose, nor do six of the seven strains of the El Tor bacillus which Willmore has placed in a separate group in view of their absorption reactions.

Certain sugars and alcohols it will be seen give little or no aid in differentiation inasmuch as they are fermented or not fermented by the majority of the strains. These are galactose, arabinose, raffinose, erythrone, adonite and possibly also glycerine and amygdalin.

Also, with five or six exceptions, all the bacilli investigated in Table I have no action on sorbite, inulin, salacin and isodulcite. That these exceptions were not due to indefinite reactions or faulty observation, however, was shown by the fact that some of these bacilli were retested after an interval of two months on the same sugars and alcohols with absolutely identical results.

Of the 25 strains in this group only seven ferment sorbite and six of these were isolated by Willmore at El Tor. They are

- B. pseudo-dysenteriae* (Willmore) 1.
- B. pseudo-dysenteriae* (Willmore) 2.
- B. Tor 44* (Willmore).
- B. Tor 167* (Willmore).
- B. 162 U.P.* (Willmore).
- B. 165 U.P.* (Willmore).

The remaining sorbite-fermenter was the bacillus of Strong.

The failure to ferment sorbite by the members of the A group is a most important feature of this group, and serves to separate it as a whole from the Group B which contains bacilli isolated solely from sources in this country.

On looking at Table II, it will be noticed that B 1 is the only culture which gives the same reactions as *B. Flexner*.

All the others not only differ from the Flexner type, but also with the exception of B 11 and B 12, differ from one another, and, what is equally remarkable, not one of them will be found to agree completely in its reactions with any bacillus in Table I.

The principal difference between most of the cultures and *B. Flexner* is their fermentation of sorbite.

It will be noticed that B 20 to B 25 are motile bacilli; this factor when we come to consider the agglutination reactions appears to be of very great importance as a distinguishing feature.

B 17 is a bacillus of some interest in that its cultural reactions on all the media are identical with those of the typhoid bacillus, differing from this bacillus only in its non-motility. Numerous attempts were made to render it motile, such as passage through a guinea-pig and subculturing on seventeen successive days on to peptone beef broth, but without success.

The cultures in Table II have been divided into groups according to their reactions on the various media on the list as far as and including dextrin. Thus the members of Group I differ from the Flexner group in that they ferment sorbite and the members of Group II in that they ferment sorbite while failing to ferment maltose or dextrin. The members of Group III differ from *B. Flexner* in that they clot milk and produce no indol. The members in Group IV differ from the Flexner type in many respects, one striking difference being their motility.

In contrast to the A group, only galactose, erythrite and adonite can be removed as affording no aid in differentiation. Considerable differences occur between the various bacilli of the B group in their reactions on arabinose, raffinose, glycerine and amygdalin. With regard to B 1 which is the only bacillus corresponding in all particulars to *B. Flexner*, it has to be noted that the child from whom it was isolated was in all probability infected by the father who had suffered from bacillary dysentery abroad. The attempts made by Marshall to demonstrate dysentery bacilli in the excreta of the father were not successful but little stress can be attached to the negative result in

view of the marked intermittency in the excretion of the specific bacilli in such cases.

B 5 and B 6 isolated from the faeces and urine respectively of the same case resemble each other very closely in their fermentation characters, the only discrepancy being a late reaction on raffinose by B 5.

B 2 and B 4 obtained from mother and daughter respectively differ only in their action on raffinose and amygdalin. They are otherwise identical in their fermentation characters.

B 3, B 8 and B 14 from members of the same household differ very considerably, two of them being unable to ferment maltose and dextrin.

B 11 and B 13 from different cases of dysentery in the same asylum are the only two bacilli in the whole group which give absolutely identical reactions on all the sugars tested. B 15 and B 16 also from the same asylum exactly resemble one another except on raffinose. B 12 cannot be considered identical with any of the others from this asylum.

#### *Agglutination Reactions.*

The next test applied was that of the agglutination reactions. Rabbits were immunised with *B. Flexner*, *Bacillus "Y"* of Hiss and Russell and *B. Strong* respectively, and each of the cultures in Tables I and II were tested with the sera, the results being set forth in Tables III and IV.

It will be observed that all the members of the Flexner group are agglutinated by Flexner serum to the same extent, with the exception *B. "Y,"* although conversely *B. Flexner* is agglutinated as well by "*Y*" serum as *B. "Y,"* itself. It will be further noticed that none of the other bacilli in Table III are agglutinated by Flexner serum in a higher dilution than 1 in 2000, with the exception of the five following, viz. A 8, or *B. pseudo-dysenteriae* A of Kruse, A 13 or *B. pseudo-dysenteriae* of Willmore, and A 22, A 23 and A 24 isolated from cases of infantile diarrhoea at Alexandria.

A 25 and A 26 are agglutinated by none of these sera. It will also be noticed that all the bacilli in this table are agglutinated in a higher dilution by "*Y*" serum than they are by Flexner serum, although these two sera are of the same titre.

The "*Strong*" serum, as might be expected from the cultural reactions of the bacillus, seems to have very little affinity for any of the bacteria in this table, with the exception of A 13 which was

TABLE III. (*Foreign Strains.*)

Designation	<i>B. Fleischer</i> serum (titre 1 in 20,000)		<i>B. "Y"</i> Hiss and Russell serum (titre 1 in 20,000)		<i>B. Strong</i> serum (titre 1 in 20,000)	
	%	%	%	%	%	%
<i>B. Fleischer</i> "T. J. P. M."	..	A 1	3	3	3	3
<i>B. Fleischer</i> "Elstree"	..	A 2	3	3	3	3
<i>B. Fleischer</i> Gray	..	A 3	3	3	3	3
<i>B. Invor</i> Baltimore	..	A 4	3	3	3	3
<i>B. "Y"</i> Hiss and Russell (Pentz)	..	A 6	3	3	3	3
<i>B. Strong</i> (Kruse)	..	A 7	0	0	0	0
<i>B. pseudo-dysenteriae</i> A Kruse	..	A 8	3	3	3	3
<i>B. pseudo-dysenteriae</i> D Kruse	..	A 9	3	3	3	3
<i>B. pseudo-dysenteriae</i> D Kruse	..	A 10	3	3	3	3
<i>B. Fleischer</i> Willmore (El Tor)	..	A 11	3	3	3	3
<i>B. pseudo-dysenteriae</i> Willmore (El Tor)	..	A 12	3	3	3	3
<i>B. pseudo-dysenteriae</i>	..	A 13	3	3	3	3
<i>B. pseudo-dysenteriae</i>	..	A 14	3	0	0	0
<i>B. Tor</i> 22 Willmore	..	A 15	0	0	0	0
<i>B. Tor R.H.B.</i>	..	A 16	3	3	3	3
<i>B. Tor R.E.T.</i>	..	A 17	3	2	0	0
<i>B. Tor</i> 44	..	A 18	3	2	0	0
<i>B. Tor</i> 12/10	..	A 19	3	3	1	0
<i>B. Tor</i> 36/7	..	A 20	3	3	0	0
<i>B. Tor</i> 167	..	A 21	3	3	3	0
<i>B. infantis</i> diarrhoea	..	A 22	3	3	3	3
<i>B. infantis</i> diarrhoea	..	A 23	3	3	3	3
<i>B. infantis</i> diarrhoea	..	A 24	3	3	3	3
<i>B. 162 U.P.</i>	..	A 25	0	0	0	0
<i>B. 165 U.P.</i>	..	A 26	0	0	0	0

N.B.—1=slight reaction,  
2=distinct reaction,  
3=complete reaction.

TABLE IV. (*British Strains.*)

B. Flavner serum (titre 1 in 20,000)		B. Strong serum (titre 1 in 20,000)	
E. "Y," Hiss and Russell serum (titre 1 in 20,000)		B. Strong serum (titre 1 in 20,000)	
B1	1 in 20000	B1	1 in 20000
B2	1 in 50000	B2	1 in 50000
B3	1 in 100000	B3	1 in 100000
B4	1 in 200000	B4	1 in 200000
B5	1 in 400000	B5	1 in 400000
B6	1 in 500000	B6	1 in 500000
B7	1 in 1000000	B7	1 in 1000000
B8	1 in 2000000	B8	1 in 2000000
B9	1 in 4000000	B9	1 in 4000000
B10	1 in 8000000	B10	1 in 8000000
B11	1 in 16000000	B11	1 in 16000000
B12	1 in 32000000	B12	1 in 32000000
B13	1 in 64000000	B13	1 in 64000000
B14	1 in 128000000	B14	1 in 128000000
B15	1 in 256000000	B15	1 in 256000000
B16	1 in 512000000	B16	1 in 512000000
B17	1 in 1024000000	B17	1 in 1024000000
B18	1 in 2048000000	B18	1 in 2048000000
B19	1 in 4096000000	B19	1 in 4096000000
B20	1 in 8192000000	B20	1 in 8192000000
B21	1 in 16384000000	B21	1 in 16384000000
B22	1 in 32768000000	B22	1 in 32768000000
B23	1 in 65536000000	B23	1 in 65536000000
B24	1 in 131072000000	B24	1 in 131072000000
B25	1 in 262144000000	B25	1 in 262144000000

*N.B.* —1 = slight reaction, 2 = distant reaction.

agglutinated by all three sera. The El Tor strains are seen to be agglutinated readily by *B. "Y"* serum but not by *B. Flexner* serum.

I may add that all the cultures in both tables were tested with normal rabbit serum, but none of them were found to be agglutinated in a higher dilution than 1 in 20 of the serum except B 15 and B 16 which were agglutinated by normal serum in dilutions of 1 in 500 and 1 in 200 respectively.

Table IV is a record of the agglutination reactions of the bacilli in Table II. Here too, as was observed in respect of Table III, all the cultures are agglutinated in higher dilutions by "Y" than they are by Flexner serum, and few of them, and those only in low dilutions, are agglutinated by "Strong" serum.

Turning to the particular cultures in this table, B 1 is agglutinated in a low dilution only (1 in 200) by Flexner serum, in much higher dilution (1 in 5000) by "Y" serum, and as might be expected from its cultural reactions, not at all by "Strong" serum.

The next cultures under consideration are those included in Group I, whose principal cultural difference from *B. Flexner* consists in their fermentation of sorbite. These are all of them agglutinated by Flexner serum but in a low dilution only. They are agglutinated by "Y" serum in very high dilutions, and either not at all, or, as in the case of B 2 and B 3, in low dilutions only by "Strong" serum.

The cultures in Group II are, for no apparent cultural reasons, again split up into two divisions by their agglutination reactions. B 7, B 8 and B 9 are agglutinated by both Flexner and "Y" sera, also to a minor extent by "Strong" serum, whereas the remaining members of this group, B 10, B 11, B 12 and B 13 failed to be agglutinated by any of the three sera, with the exception of a complete reaction given by B 13 in a dilution of 1 in 200 of "Y" serum.

B 14, it will be observed, is agglutinated by none of these sera.

The two cultures in Group III are agglutinated by all three sera, but especially well by "Y" serum. B 17 is also agglutinated by none of the sera, but, owing to its great resemblance culturally to the typhoid bacillus, its agglutination reactions were further tested with typhoid serum of a titre of 1 in 20,000, by which it proved to be agglutinated in a dilution of 1 in 5000.

B 18 and B 19, both cane-sugar fermenters, and in this respect resembling the bacillus of Strong, gave no reaction with either Flexner or "Y" serum, but are agglutinated in a low dilution of 1 in 200 only by Strong serum.

The members of Group IV, being all motile bacilli, are none of them agglutinated by any of the three sera, nor are they agglutinated by typhoid serum in even as low a dilution as 1 in 20.

From these results it will be seen how impossible it is, judging these atypical bacilli by their cultural reactions alone, to say whether they will be agglutinated by Flexner serum or not. Motility, however, appears to be a complete bar to their being agglutinated by this serum, as is shown by the cultures in Group IV.

B 5 and B 6, which resembled each other closely on the various sugars, are also alike in their agglutination reactions with the three sera.

B 2 and B 4 are also alike in their agglutination characters.

B 3 and B 8 agree closely in their agglutination characters but B 14 is not agglutinated by any of the three sera.

B 11 and B 13 are neither of them agglutinated by Flexner serum but B 13 is agglutinated up to 1 in 200 by "Y" serum. B 11 is not agglutinated by "Y" serum.

#### *Absorption experiments.*

The next test applied to these cultures was that of completely absorbing Flexner and "Y" sera respectively, with each of these cultures in turn. For this purpose a sufficient quantity of the culture was added to 1 in 100 dilution of the serum to ensure complete absorption of all the specific agglutinins for that bacillus, so that after centrifugalising the bacilli, the clear serum no longer agglutinated that bacillus. The supernatant fluid was then tested with the homologous bacillus in 1 in 200, 1 in 500, 1 in 2000 and 1 in 5000.

In making these experiments with Willmore's cultures I confined myself to the use of "Y" serum only, since Willmore had already demonstrated by means of absorption experiments that his cultures could be distinguished from *B. Flexner*, and I thought it unnecessary to repeat his experiments. The results are set forth in Table V.

It will be seen that two of Willmore's strains of El Tor bacilli A 19 and A 20 can be differentiated from *B. "Y"* by these absorption experiments.

*B. Flexner* (Gray) is shown to be identical with *B. Flexner* (Philippines) from which the serum was made, but *B. Flexner* when made to absorb "Y" serum can be readily differentiated from that bacillus. A 8 and A 9, *B. pseudo-dysenteriae* A and *B. pseudo-dysenteriae* D of

*B. dysenteriae*

Kruse, are similarly distinguished from *B. "Y"* as also is A 22 a bacillus isolated from infant's diarrhoea at Alexandria.

Absorption experiments with fifteen strains which were found to be agglutinated by Flexner or "Y" serum were tested by means of absorption experiments with Flexner and "Y" serum. The results are set forth in Table V. On examining this table it will be seen that none of these tests gave a definitely positive result, with the exception of that applied to B 2 which absorbed all the agglutinins both from Flexner and "Y" serum for their homologous bacilli. B 4, B 5, B 6 and B 8 only effect a partial absorption of the Flexner-agglutinins.

TABLE V. *Absorption Experiments.*

	Still agglutinates <i>B. Flexner</i>			"Y" serum (titre 1 in 20,000)			Still agglutinates <i>B. "Y"</i>			
	1 in 200	1 in 500	1 in 2000	totally absorbed by the following bacilli	1 in 200	1 in 500	1 in 2000	1 in 5000		
A 3 ( <i>Flexner Gray</i> )	0	0	0	A 1 ( <i>Flexner</i> )	...	3	3	3	3	
	...	...	:	A 8 ( <i>Pseudo-dys. A Kruse</i> )	3	3	3	3		
	...	...	:	A 9 ( <i>Pseudo-dys. D Kruse</i> )	3	3	3	3		
	...	...	:	A 19 ( <i>B. Tor</i> )	...	3	3	3	3	
	...	...	:	A 20 ( <i>B. Tor</i> )	...	3	3	3	3	
	...	...	:	A 22 ( <i>Infant's Diarrhoea</i> )	3	3	3	3		
B 1	...	...	3	B 1	...	...	3	3	3	3
B 2	...	...	0	B 2	...	...	0	0	0	0
B 3	...	...	3	B 3	...	...	3	3	2	1
B 4	...	...	3	B 4	...	...	3	3	3	1
B 5	...	...	3	B 5	...	...	3	3	3	0
B 6	...	...	3	B 6	...	...	3	3	3	0
B 7	...	...	3	B 7	...	...	3	3	3	0
B 8	...	...	3	B 8	...	...	3	3	3	3
B 9	...	...	3	B 9	...	...	3	3	3	1
B 15	...	...	3	B 15	...	...	3	3	3	1
B 16	...	...	3	B 16	...	...	3	3	3	0

On referring to Table II, B 2 will be found in Group I, and it will be observed that its cultural reactions differ from *B. Flexner* and *B. "Y"* owing to its fermentation of sorbite, amygdalin and isodulcite, so that although this bacillus appears to be capable of producing the same agglutinins as *B. Flexner* or *B. "Y"*, it can readily be distinguished from them by its cultural reactions.

*Test of cultures giving similar agglutination reactions.* (Table VI.)

From the above results the question arises as to whether all the cultures in Table IV which either are, or are not, agglutinated by Flexner serum are between themselves identical. It was found that

12 of these cultures were agglutinated and that 8 of them were not agglutinated by Flexner serum, consequently in order to solve this problem a culture from each of these two groups was selected, and rabbits were immunised with them. B 9 was selected from amongst the former and B 11 from amongst the latter as shown in Table VI. The agglutination reactions of each member of the groups were then tested with these sera.

Of those tested against B 11 serum, *i.e.* the bacilli that are not agglutinated by Flexner serum, only two were found to be agglutinated by this serum. These were B 12 in a dilution of 1 in 200, and B 13 in a dilution of 1 in 400. On referring to Table II these two cultures

TABLE VI.

*Bacilli that do not agglutinate with  
Flexner serum.*

B 11 serum, prepared from one of  
their number.

*Titre of serum 1 in 20,000.*

No.	1 in 20	1 in 50	1 in 100	1 in 200	1 in 400	1 in 800	1 in 1600	1 in 3200	1 in 6400	1 in 12800	1 in 25600
B 10	0	0	0	0	0	0	0	0	0	0	0
B 12	3	3	3	2	0	0	0	0	0	0	0
B 13	3	3	3	3	2	0	0	0	0	0	0
B 14	0	0	0	0	0	0	0	0	0	0	0
B 17	0	0	0	0	0	0	0	0	0	0	0
B 19	0	0	0	0	0	0	0	0	0	0	0

*Bacilli that agglutinate with  
Flexner serum.*

B 9 serum, prepared from one of  
their number.

*Titre of serum 1 in 2000.*

No.	1 in 20	1 in 50	1 in 100	1 in 200	1 in 400	1 in 800	1 in 1600	1 in 3200	1 in 6400
B 1	3	3	3	3	3	3	3	2	1
B 2	2	2	0	0	0	0	0	0	0
B 3	3	3	3	3	3	3	2	1	1
B 4	0	0	0	0	0	0	0	0	0
B 5	0	0	0	0	0	0	0	0	0
B 6	0	0	0	0	0	0	0	0	0
B 7	0	0	0	0	0	0	0	0	0
B 8	3	3	3	3	3	3	3	3	3
B 9	3	3	3	3	3	3	3	3	2
B 15	3	3	3	3	2	1	0	0	0
B 16	3	3	3	3	1	0	0	0	0
B. Flexner	3	3	3	2	0	0	0	0	0
B. "Y"	3	3	3	2	0	0	0	0	0

*Absorption Experiments.*

B 11 serum totally absorbed by the following bacilli	Still agglutinates B 11			
	1 in 200	1 in 500	1 in 2000	1 in 5000
B 12	3	3	3	3
B 13	3	3	3	3

B 9 serum totally absorbed by the following bacilli	Still agglutinates B 9			
	1 in 50	1 in 200	1 in 500	1 in 2000
B 1	3	3	3	3
B 3	0	0	0	0
B 8	0	0	0	0
B 15	3	3	3	1
B 16	3	3	3	1
B. "Y"	3	3	2	0
B. Flexner	3	3	3	0

are seen to be somewhat alike in their cultural reactions both being members of Group II.

The next experiment was to test those bacilli which do agglutinate with Flexner serum, against B 9 serum. The result was that six of them, B 1, B 3, B 8, B 9, B 15 and B 16, were found to be agglutinated fairly well by this serum whereas on testing *B. Flexner* and *B. "Y"* with this serum, the titre of which was 1 in 2000, they were found to be agglutinated in no higher dilution than 1 in 400.

Absorption experiments were then made with these two sera in a similar manner to that previously described. By this means B 12 and B 13 were distinguished from B 11, for although they were agglutinated by B 11 serum they failed to absorb all the agglutinins from that serum for its homologous bacillus.

Similar absorption experiments were made with B 9 serum in respect of those bacilli which were found to be agglutinated by it, that is with B 1, B 3, B 8, B 15 and B 16. As a result of these experiments, B 3 and B 8 appeared to be identical with B 9, that is, as far as absorption tests go, although, on referring to Table II, it will be noticed that these two bacilli are very different culturally.

From the results of all these experiments with the cultures in Table II, it will be seen that culturally only B 1 conformed to the Flexner type, and that although this bacillus is agglutinated both by Flexner and "Y" serum, it is differentiated from these bacilli by the absorption tests. Judging by the absorption test alone B 2 is the only culture which appears to be identical with the Flexner group but its cultural reactions differ widely from those of the Flexner bacilli.

#### *Test of Virulence.*

The virulence of some of these cultures was tested by injecting 0.5 c.c. of a 48 hours' broth culture intraperitoneally into guinea-pigs. The cultures selected were B 1, B 2, B 3, B 7, B 14 and B 21. All the animals died within 36 hours with the exception of the one injected with B 21, the motile culture from a sausage; this animal remained unaffected.

#### *Analysis of Results.*

It will be convenient to discuss separately the results obtained with the strains from the foreign and the home sources. With regard to the former, out of a total of 25, 22 are agglutinated by "Y" serum. Of

the 22, 12 are also agglutinated in moderately high dilutions (higher than 1 in 1000) while 10 are agglutinated by this serum only in dilutions of 1 in 400, and under (1 in 20 to 1 in 400). Thus the "Y" serum has a far more powerful effect on the members of the mannite-fermenting dysentery group generally than Flexner serum.

The employment of "Y" serum rather than Flexner serum for agglutination purposes would therefore be of distinct advantage in settling the broad question of admission to the mannite-fermenting group.

The strains which responded neither to Flexner nor "Y" serum were *B. Strong* and two of Willmore's Arabian strains. The fact that *B. Strong* will not react to Flexner or "Y" serum is, however, well known and it is of interest that a Strong serum has little action on any of the bacilli tested except the homologous one. Certainly one strain A 13 from El Tor, identified by Willmore with the *B. pseudo-dysenteriae* of Kruse, is agglutinated in equally high dilutions by Flexner, "Y" and Strong serum, while three other strains are agglutinated in dilutions varying from 1 in 50 to 1 in 400; so that after all, a Strong serum though almost specific is not absolutely so for bacilli of the Strong type. The two strains of Willmore (A 23 and A 29) which did not react towards any of the three sera must be left out of consideration in the meantime. A satisfactory explanation for their failure to react cannot at present be suggested. That both ferment sorbite is of interest, but on the other hand A 13, A 14 and A 21 (also Arabian strains), which likewise ferment sorbite, readily react to Flexner and "Y" serum.

When we come to consider the fermentation reactions of these bacilli it has to be remembered that in most papers dealing with this subject an extensive series of sugars, alcohols and glucosides has not been tested. As a rule, only glucose, mannite, cane-sugar, maltose and dextrin have been called into requisition in the differentiation of the well-known types. With the large number employed in the above work it is seen that only the members of the Flexner group and the bacillus "Y" (at least the particular strains tested) agree in all particulars on the whole series of sugars. With these exceptions, all the organisms differ in certain particulars although it must be admitted that these differences in some cases are slight.

Even a casual glance at Table I exhibits a much greater uniformity among the strains of the "A" group than exists among the strains of the "B" group. Nevertheless the fact that only the Flexner strains remove Flexner agglutinins while the other (six) "A" strains tested

failed to remove the homologous agglutinins from "Y" serum, suggests that the fermentation variations, insignificant though they appear to be at first sight, are of real importance and are correlated in some way with the variations in the receptor mechanism as evidenced by the absorption results. Turning to the second group "B" we find that one bacillus only, viz. B 1, corresponds throughout in its fermentation characters with *B. Flexner*, whereas all the others differ in certain particulars from each other and from the members of the "A" group. In regard to the agglutination tests the strains B 20 to B 25, being frankly motile bacilli and entirely uninfluenced by either Flexner or "Y" serum, may be at once excluded from the dysentery group. That they were also uninfluenced by typhoid anti-serum has already been mentioned. Of the remaining 19 strains no fewer than 11 are agglutinated by "Y" serum in dilutions varying from 1 in 2000 to 1 in 20,000, and one is agglutinated in a dilution of 1 in 200 only.

On the strains of this group also, Flexner serum has a less powerful influence than "Y" serum. On the strength of the agglutination tests we must admit these 11 strains and possibly also B 13 to membership of the mannite-fermenting dysentery group, and on referring to the source of these strains we find that eight of them came either from apparently healthy persons or from persons in late convalescence from typhoid fever in whom either no history at all, or no satisfactory history, of clinical dysentery could be obtained. It is remarkable that B 3 and B 8 isolated from two members of the same family give somewhat different fermentation reactions but the same phenomenon is apparent in the case of the strains from the same asylum (Claybury).

Of the Claybury strains it would appear from agglutination reactions that B 15, B 16 and possibly B 13 should be admitted to the mannite-fermenting group, but a further examination of the two former, viz. B 15 and B 16, showed that they were influenced quite as highly by an anti-typhoid serum. Moreover, in addition to their ready agglutinability, the fact that, like Strong, both these strains ultimately clotted milk, will not permit us to include them with any confidence in this group. On the other hand it has to be observed that B 11 although absolutely identical with B 13 in its fermentation reactions is not agglutinated at all by Flexner or "Y" serum. Nor is B 12, which however differs considerably from the other strains from this asylum in its fermentation properties.

We cannot definitely reject B 11 and B 12 from the mannite-fermenting dysentery group, however, in view of the fact that a serum

prepared by immunising with B 11 agglutinates the two strains B 12 and B 13, the latter of which is agglutinated by a "Y" serum though admittedly in a low dilution. The question must remain at present unsettled as we may be dealing with strains which are only with difficulty agglutinable by any serum.

Of the remaining strains, B 10 isolated from a mouse must be rejected from the group in question, also B 14, B 18 and B 19. With regard to the two latter, B 18 and B 19 isolated from urine of two typhoid convalescents, it is of some interest to note that both are cane-sugar fermenters and it might naturally be expected that they would give some reaction with a "Strong" serum. As a matter of fact they do agglutinate with the serum but in no higher dilution than 1 in 200, and in view of the somewhat doubtful position of the "Strong" strain itself little stress can be laid on the coincidence.

B 17 requires little notice. It agglutinates in high dilutions with a typhoid anti-serum and must be chronicled as one of the rarely encountered non-motile forms of this bacillus. Ernst (1908) and Fischer (1909) have recently reported on two such strains.

The absorption experiments carried out with this group show that five strains only, viz. B 2, B 4, B 5, B 6 and B 8, contain agglutinin-receptors more or less closely allied to those of *B. Flexner* while one only, viz. B 2, removes entirely the "Y" agglutinins from a "Y" serum. B 12 and B 13 do not remove the homologous agglutinins from B 11 serum and therefore one would again hesitate to include these bacilli in the manna-fermenting dysentery group. Further investigation is necessary.

Again B 3 and B 8 appear as the result of absorption tests to be identical with B 9. Now B 3 and B 8 were isolated on the same day from the faeces and urine respectively of two members of the same family, one of whom was alleged to have had dysentery four years ago, and yet these two bacilli differ in several particulars when their fermentation characters are compared.

It is clear, therefore, that fermentation tests have indicated differences in individual strains which were not brought out by the absorption method. So far as the absorption tests above are concerned, these go to show that comparatively few of the strains in question, whether of foreign or home origin, can be considered identical and that in the few cases where such identity is apparently established the fermentation results on an extended series of sugars may still exhibit small but decided differences.

From the practical point of view the absorption method is a laborious one and in my experience with this group has failed to indicate a similarity between the strain tested and the various well-known types. The fermentation properties have in most cases confirmed such dissimilarity and have further indicated differences which the absorption tests mask.

In the mannite-fermenting dysentery group (excluding the "Strong" strains) must be incorporated a large and probably ever-increasing number of strains reacting with striking uniformity to one test (*i.e.* agglutination with "Y" or Flexner serum), but differing from one another markedly when their fermentation properties and receptor mechanisms are minutely investigated.

List of strains which the results of the present research permit us to include in the mannite-fermenting dysentery group (Flexner-*"Y"* types):

B 1	B 5	and possibly B 11, B 12 and B 13.
B 2	B 6	
B 3	B 7	
B 4	B 8	
B 9		

Strains which like "Strong" clot milk and are readily agglutinable by any anti-serum:

B 15}  
B 16}.

Strains which do not clot milk but which like "Strong" ferment cane-sugar and are agglutinated slightly by a "Strong" serum:

B 18}  
B 19}.

The exact position of B 15, B 16, B 18 and B 19 cannot at present be definitely settled.

#### CONCLUSIONS.

1. A large number of dysentery-like strains isolated from sources not obviously connected with clinical dysentery in this country have been thoroughly tested and a certain proportion of them has been found to be entitled to membership of the mannite-fermenting dysentery group.

2. Bacillary dysentery must be more widely distributed in this country than has hitherto been believed and thorough bacteriological investigation may throw light on the aetiology of dysenteric forms of diarrhoea which at present are not clinically recognised as such.

3. Like certain foreign strains recently isolated from definite dysenteric sources, these home strains cannot be identified completely

with any of the well-known types of the group, on application of extensive fermentation and absorption tests.

4. When a sufficiently extended series of carbohydrate media is tested the fermentation properties of the mannose-fermenting group afford an indication of differences between the members of the group, which are not brought to light by agglutination and absorption tests.

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## AN OUTBREAK OF ACUTE GASTRO-ENTERITIS CAUSED BY *B. PARATYPHOSUS* (B.).

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### *History of the Outbreak.*

ON June 14th, 1910, the attention of one of us was directed by the House Physician of the Hampstead General Hospital, to an outbreak in Paddington of what was thought to be "food-poisoning." Two of the patients were at the time under his care.

The history of the outbreak was briefly as follows: On the night of the 10th—11th June, eight persons out of twenty-nine living in a high-class boarding-house were taken ill, five of the cases being amongst the servants, seven in number, and three among the guests, twenty in number. The two proprietors of the house were not affected. All the patients were females and with one exception (aetat. 15) adults, some of mature age.

The symptoms were practically the same in all the cases, but one patient was much less affected than the rest. She had, by coincidence, taken a purgative on the night of the 9th and her escape from an attack of a severity equal to that of the others may be attributed to that cause. All had severe abdominal pain, followed by vomiting and persistent and violent diarrhoea, which could not be controlled by the usual remedies. Fever supervened, the temperature rising as high as 103° F. in some cases, and lasting for various periods up to a week. There were no indications of any pulmonary lesions, or of any splenic enlargement, nor was there any delirium. Some of the more elderly

patients had attacks of grave syncope, and, with the exception of the case already mentioned, convalescence was slow. All the patients recovered.

The first inquiries were naturally directed to find out what food had been the cause of the outbreak. The time of the onset of the outbreak and the fact that none of the male guests had been attacked suggested that the infected food—if there had been any—had been eaten at the midday meal of the 10th at which only one male guest had been present. Careful inquiries, however, failed to elicit any evidence of any item of the menu of that meal having been taken by the patients and passed by those who escaped. Still, in view of the well-known capriciousness manifested in the attack and escape of persons partaking of infected food, it was thought advisable to pursue inquiries with relation to each article of food provided at the meals of the 9th and 10th. The results of the inquiries were entirely negative and while they were in progress the bacteriological examinations, which are described below, led us to the conclusion that food infection had not been operative.

Samples of the faeces from two patients were received on the 14th and forwarded to the Lister Institute, the patients then having been three days ill. It was not known at the time that one of the patients had been treated with saline irrigations of the rectum, but that information subsequently explained the fact of her faeces yielding practically no growth. The specimens when received were quite fluid and of a brilliant green colour. A third sample was received on the 17th from a patient then ill six days, who had had 14 or 15 motions on that day. This patient (Miss L.) was upwards of 70 years old, and hers was one of the severest cases of the outbreak. She had several attacks of heart failure, and her case gave rise, at one time, to much anxiety.

Specimens of blood were obtained from six of the patients and submitted to agglutination tests at the Lister Institute.

In the course of the investigation, information was received of similar cases in Willesden, Hampstead and Lambeth which were thought to have been connected with the Paddington outbreak. Details of those cases were obtained from the Medical Officers of Health of the districts named and such information is summarised in the following paragraphs:—

(1) A group of cases occurred in the family (named H.) of the temporary cook at the boarding-house, four persons being taken ill during the morning of the 14th. All had abdominal pain and diarrhoea, but

vomiting was not present in all cases. There was a history of dried haddock being eaten at supper on the night of the 13th but, in the light of other evidence, the causal relationship between the fish and the illness appears to be doubtful. Specimens of the blood from two of the patients (Mr and Miss H.) were submitted to agglutination tests.

(2) A laundry proprietor, named T., also residing in Willesden, who called at the boarding-house on the 10th, had diarrhoea on that and the following two days, which he attributed to eating a veal and ham pie on the 10th. There was no history of any illness among the other members of T.'s family.

(3) A further case in the same district was reported in the person of a woman who was at the time of the outbreak kitchen-maid at the boarding-house, but, as the woman was said to have taken a purgative just before her attack of diarrhoea, the case was not (unfortunately) followed up.

(4) The case in Hampstead was in the person of a married sister (Mrs R.) of the temporary cook at the boarding-house, with whom the cook lived. Mrs R. visited her mother Mrs H. in Willesden on the 13th and the same evening had severe abdominal pain, followed in the morning by vomiting and diarrhoea. Mrs R. did not share the haddock supper to which the H.'s attributed their illness, but she had a piece of cold fried fish brought from the boarding-house on the previous Sunday (12th). An unavailing attempt was made to secure a specimen of Mrs R.'s blood.

(5) The Lambeth case was that of Mrs D. who had been married only a short time, leaving a situation in the boarding-house for that purpose. She visited the house on Monday June 27th to see one of the servants, had "tea" in a bedroom in which one of the original patients had been nursed and had an attack on the following day, which is best described, in the absence of medical information, in her own words. She wrote to her friend: "I have been so ill since I saw you on Monday. All day Tuesday and Wednesday I felt as though I was going to die. I was sick and all the other (*sic*) all day and night, doubled up with pain and all that came up was like thick green paint." Such account suggests that Mrs D. had a pretty severe attack of diarrhoea and vomiting, much the same as the inmates of the boarding-house. The Medical Officer of Health of Lambeth reported that Mrs D.'s medical attendant described the case as one of ptomaine poisoning. The "tea" referred to is said to have consisted of tea and bread and butter—no potted meats. A specimen of Mrs D.'s blood was refused.

*Bacteriological Examination.*

(a) Three samples of faeces were received during the outbreak, two on June 15th from Miss G. and Miss W. and one on June 17th from Miss L. When examined by the methods usually employed for such cases, the faeces of Miss W. yielded no growth, but the faeces of Miss G. and Miss L. yielded cultures which were identical in their cultural characters with *B. paratyphosus* (B.). The nature of these cultures was determined by observing their agglutination and absorption reactions with various sera. The results which are given in Tables I and II show that both the strain "G" from Miss G., and the strain "L" from Miss L. were indistinguishable from standard strains of *B. paratyphosus* (B.).

These results show that the bacilli isolated from the patients' faeces were *B. paratyphosus* (B.). Strain G was found to be highly virulent

TABLE I. *Agglutination tests.*

The Macroscopic method was used, and the agglutination limits were observed after incubation for 2 hours at 37° C. Controls were made in every case.

Organism	Serum		
	<i>B. paratyphosus</i> (B.)	<i>B. suis</i> <i>pestifer</i>	<i>B. Gaertner</i>
<i>B. paratyphosus</i> (B.), McWeeney	20,000	5,000	<100
<i>B. suis</i> <i>pestifer</i> (Laboratory strain, Lister Institute)	5,000	10,000	<100
Strain G 1	20,000	5,000	<100
Strain G 2	20,000	5,000	<100
Strain L	20,000	5,000	<100
<i>B. Gaertner</i> (original strain of v. Ermengen)	<100	<100	5,000

TABLE II. *Absorption tests.*

Macroscopic method. Agglutination limits after absorption, observed after incubation for 2 hours at 37° C. Controls made in every case.

Serum	Organisms			
	<i>B. suis</i> <i>pestifer</i>	<i>B. paratyphosus</i> (B.)	Strain G	Strain L
I. <i>B. suis</i> <i>pestifer</i> . Original titre	5,000	5,000	5,000	5,000
<i>B. suis</i> <i>pestifer</i> . Absorbed with <i>B. paratyphosus</i> (B.)	2,000	<100	<100	<100
II. <i>B. paratyphosus</i> (B.). Original titre	5,000	20,000	20,000	20,000
<i>B. paratyphosus</i> (B.). Ab- sorbed with <i>B. suis</i> <i>pestifer</i>	<200	10,000	10,000	—

for guinea-pigs, since 1/1000 c.c. of a 24-hour broth culture, injected intraperitoneally, killed in three days.

The faeces of patients G. and L. were filtered through a Berkefeld filter, and a dose of 2 c.c. of the filtrate was injected into a guinea-pig without producing any obvious effect.

(b) Blood was obtained from six of the patients at intervals of 10—20 days after the onset of the symptoms and agglutination tests were carried out for strain G., *B. suispestifer* and *B. paratyphosus* (B.). It will be seen from Table III that, with one exception, all the patients' blood agglutinated these three organisms and as a rule to the same extent.

The serum of the patients was pooled, and the combined serum was absorbed with *B. suispestifer* and *B. paratyphosus* (B.) and its agglutination limits after absorption were ascertained (Table IV).

TABLE III. *Agglutination tests carried out with the patients' sera.*

Wright's method; controls were made in every case.

Patients' serum	Organism		
	<i>B. paratyphosus</i> (B.)	<i>B. suispestifer</i>	Strain G
(1) Miss G. 1st sample	1—200	1—200	1—200
(2) " 2nd "	1—400	—	1—400
(3) B. B.	1—400	1—400	1—400
(4) Miss W.	1—200	1—200	1—200
(5) Mr H.*	1—400	1—400	1—400
(6) Miss H.*	1—400	1—400	1—400
(7) Mrs W.	1—100	1—100	1—100
(8) Mr S.	—	—	<1—40
(9) Miss L.	<1—200	<1—200	<1—200

\* These patients resided at Willesden, not at the boarding-house.

TABLE IV. *Pooled serum. Absorption test.*

Wright's method. Agglutination limit after absorption, observed after incubation for 2 hours at 37° C. Controls made in every case.

Pooled serum.	Serum	Organisms		
		<i>B. suispestifer</i>	<i>B. paratyphosus</i> (B.)	Strain G
(1) Original titre		100	100	100
(2) After absorption with <i>B. suispestifer</i>		<20	80	80
(3) After absorption with <i>B. paratyphosus</i> (B.)		<40	<40	<40

## CONCLUSIONS.

It thus appears that, while the bacteriological evidence pointed to only one organism, viz. *B. paratyphosus* (B.), as the cause of the infection in all cases, no one article of food had been eaten by all the patients and the only link between the primary outbreak in Paddington and the secondary cases in Willesden, Hampstead, and Lambeth, was furnished by the exchange of visits between the different houses. A consideration of these facts made it clear that the outbreak was almost certainly not caused by food, and an attempt was made to find a paratyphoid-carrier. The existence of paratyphoid (B.) carriers is now well known (Lentz [1905], Prigge and Sachs-Muke [1909]), and there are grounds for supposing that this organism has its habitat in the human alimentary canal. It was impossible, however (as is often the case in such outbreaks), to obtain the necessary material (blood and faeces) from those members of the boarding-house, who had not been ill, and no actual carrier was found.

For a time suspicion fell upon a servant, who had entered the boarding-house only a few days before the outbreak took place, and who had remained at work during the outbreak (although she had a mild attack of diarrhoea); but the examination of her urine and faeces always yielded a negative result as regards *B. paratyphosus* (B.) although her blood still agglutinated this organism up to 1—100 four months after the outbreak.

Nevertheless, the outbreak is of interest for two reasons. In the first place, it is clear that, as Schottmüller (1904) pointed out, *B. paratyphosus* (B.) is capable of giving rise not only to paratyphoid fever, but also to acute gastro-enteritis simulating "food-poisoning," a fact not hitherto observed in this country. Secoundly, the distribution and dates of onset of the illness of the various cases were unlike those of ordinary "food-poisoning," and pointed to a human source of infection.

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## STUDIES IN BACTERIAL VARIATION.

WITH SPECIAL REFERENCE TO THE CHEMICAL FUNCTIONS  
OF THE MEMBERS OF THE TYPHOID-COLI GROUP.

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With Plate I, and 9 Charts.

MUCH recent work has demonstrated the occurrence of variation in the fermentation properties of intestinal bacteria. As is well known these functions are of great service in the differentiation of species and varieties and accordingly any evidence of instability on their part assumes at once considerable practical importance. These changes may show themselves by (1) the acquirement of a new fermentation character, or (2) by the loss of such a character, or the two processes may go on simultaneously. The acquirement of such a new character may be sudden or gradual.

The sudden changes have received particular attention since M. Neisser (1906) described a case of mutation, in which a certain coliform organism took on the lactose-fermenting power suddenly and retained that power with great tenacity. This organism was subsequently very fully investigated by Massini (1907), who demonstrated convincingly that the new lactose-fermenting organism was in reality derived from the non-lactose-fermenting ancestor and also that it was absolutely stable. On lactose-fuchsin-agar it was found to form white colonies, but from the second day onwards papillae (Knöpfe) developed on those colonies which were not crowded together. The papillae were white at first, becoming red later. They appeared only if milk sugar was present, a concentration of 0.1% of this sugar being sufficient to

produce them. Sub-cultures from the white colonies, within 24 hours of their appearance, gave only white colonies on a lactose-fuchsin plate. These colonies remain white, but develop red papillae. Sub-cultures from the papillae, however, give both red and white colonies, and from the former red colonies only appear on sub-culture. Papillae never form on them. The red strain remained constant during many months of culture apart from lactose, and Massini after trying many devices found the new characters could only be removed by growing the organism in carbolic acid media. This fact, however, does not conflict with the idea of the absolute stability of the new character, as the addition of carbolic acid has been shown to destroy the natural fermenting power of many organisms. An antiserum made by immunising with the white strain agglutinated red and white organisms equally highly and a similarly prepared serum for the red organisms acted equally on both.

It appeared, therefore, that a new type of organism had arisen incapable of papillae-formation on lactose but capable of fermenting this sugar.

Subsequently Burk (1908) described a similar organism, but its serological reactions were not quite so satisfactory as in Massini's case. Reiner Müller (1908-1909) described a number of instances in which organisms behaved in an analogous manner, all of which he interprets as being of the nature of mutation. From faeces he isolated two non-lactose fermenters, colonies of which developed red papillae on litmus lactose agar. Sub-cultures from these papillae fermented lactose. Three other strains isolated from faeces formed papillae on arabinose, but further sub-culture on this medium was attended by loss of this papillae-forming power, although a more vigorous growth resulted. One of these three strains in the process of variation lost the power to ferment galactose which it formerly possessed. Fifty strains of *B. typhosus* were found to produce these papillae on isodulcite, and sub-culture from the papillae gave, in each case, colonies (1) capable of papillae-production, (2) incapable of further papillae-production and either able or unable to ferment isodulcite.

*B. dysenteriae* (Flexner type) behaved in the same way on isodulcite. *B. paratyphosus* (B.) also produced similar papillae on raffinose. Reiner Müller concluded from his work that these mutation-processes are not at all infrequent and that they are specific, i.e., take a definite direction for certain definite well-defined organisms. He worked with many organisms, but not in so thorough a manner as Massini, and in respect of

constancy of the new types, and absence of atavism, his work affords us no convincing evidence. Such constancy is essential before the process can reasonably be called mutation in the sense of de Vries. The only evidence he gives of mutation, in many cases, is papillae-formation; he does not sufficiently establish the constancy of the new types, and my own work leads me to conclude that the formation of papillae is not sufficient evidence, in itself, of such constancy.

The formation of papillae (Knöpfe) had been described before by Preisz (1904) and Eisenberg (1906). The former noted it in the case of anthrax colonies on ordinary nutrient agar of faintly alkaline reaction. The secondary colonies appear as papillae on the surface of the colony, and are hemispherical, smooth, and whitish to yellow or yellowish brown. They give the appearance to the colony of a foreign organism having grown as a contamination. After weeks or months these secondary colonies may themselves develop papillae, *i.e.*, tertiary colonies.

The anthrax papillae arise from proliferation of spores, since asporogenic races do not give them and heating at 65°C. for an hour does not prevent their appearance. Moreover if the strain is an early spore-bearer, the papillae appear early, and if late, they appear late.

Marked differences are found in the morphology of the bacilli belonging to the primary and secondary colonies. No evidence is given, however, of any new transmissible character having been acquired.

Preisz also describes, briefly, secondary colonies on cultures of *B. diphtheriae*, *B. cholerae* and *V. Finkler-Prior* and attributes them to the selection of stronger and more resistant individuals.

Eisenberg (1906) described granulations on the deep aspect of typhoid, and other colonies, on the addition of egg albumin to the medium. They appear in about two days and are of small size. The early ones coalesce, while the later ones appear near the margin of the colonies. He could not get this appearance on early cultures on ordinary agar, but in old cultures they very occasionally appeared in the case of *B. typhosus* and *B. dysenteriae*. He believes they are due to the detachment from the colonies of bacilli which penetrate more deeply into the medium and start small adjacent colonies. They are moved in the case of motile organisms by their own power and in the case of non-motile, he believes by diffusion. The diffusion of motile anaerobes through agar from a stab culture in the cold room has been demonstrated by Koninski (1902), so that the possibility of such diffusion has to be considered.

The fact that the granulations might occasionally appear in old agar cultures after some weeks (typhoid and dysentery) but never in young cultures, does not suggest that the granulations are an accompaniment of vigorous growth.

Serkowski (1901) has described daughter colonies but they are simply the cell conglomerates of which he finds bacterial colonies in general are built up. The chemical composition of the medium was not a determining factor. His secondary or daughter colonies have no resemblance to the structures we are dealing with. On the other hand, Eisenberg's granulations may very reasonably be analogous to Massini's papillae in so far as an adaptation to food supply may be the moving factor in each case.

With regard to gradual changes in the fermentation properties of the typhoid-coli group the most interesting results have been obtained by Hiss (1904) and Twort (1907). Twort found he could make *B. typhosus* ferment dulcite and lactose by simply growing it on the carbohydrate he wished it to ferment, for long periods with fortnightly sub-cultures. The method is similar to that of Neisser, but was employed for a much longer period. These sub-cultures were observed for a month. In the same way he caused *B. dysenteriae* (Shiga-Kruse) and *B. dysenteriae* (Flexner) to ferment saccharose, *B. dysenteriae* (Shiga-Kruse) to ferment lactose and the paratyphoid organisms to ferment saccharose.

Twort gives us no idea of the different lengths of time selection had to be carried on to secure his new varieties. There seems to be a good deal of evidence to show that this factor is related to the permanency of the new characters, this information would be of great value.

His successful variations caused him to regard the sugar tests as an impossible means of grouping the intestinal organisms. Reiner Müller, on the other hand, suggests that the power to vary in particular directions must be looked upon as an additional means of identification.

The principle at the basis of these variations is stated by Twort to depend on selection of individuals inclined to use the particular carbohydrate, when the other constituents of the medium are used up. This cannot be the case in the group of so-called mutations, as the change occurs when the medium is still far from being exhausted. Both groups have adaptation to food supply as a common feature, and this factor is not improbably operative in the case of Eisenberg's granulation-

formers. In the rapid cases, however, the determining cause is almost certainly the presence of inhibiting agents which hinder growth on the particular medium, but do not prevent it so powerfully on a food supply of another character. I shall go into this question more fully at a later date, but the work of Eijkmann (1904) and Konradi (1904) has shown that the arrest of growth in cultures is brought about by inhibiting agents, rather than by exhaustion of food supply. My observations confirm this. Goodman (1908) obtained some results by artificial selection in the case of the diphtheria bacillus, which are worth mentioning. By artificial selection of the highest and lowest acid-formers in a series of dextrose broths, and inoculation of a double series from them, repeating the process 36 times, Goodman obtained a strain producing intense fermentation of dextrose and another strain not fermenting it, but actually rendering the broth more alkaline. The titrations were made in each case after three days' growth at 37° C. In each "higher" series the tube of highest acidity was selected, in each lower series the lowest. He determined the acid-forming power by titration with 1/20 N. soda. It is remarkable that the variation went on in opposite directions about equally quickly. Goodman concluded that the fermenting character is a poor guide to determine whether an organism is pathogenic or merely a harmless saprophyte. The difference he obtained was as great as the natural difference between a *B. diphtheriae* and *B. pseudo-diphtheriae*. Morphological differences between the two were not marked. The greater-acid producing strain grew more vigorously than the lower one on Loeffler's serum, during the first 24 hours. The influence of this selection upon the power to ferment dextrin was negligible, both were diminished in fermenting power for maltose, and in both cases the capacity to ferment saccharose was enhanced.

Pathogenicity tests were few, but such as were given show that the greater the fermenting power, the greater is the virulence. It is remarkable that continuous growth on dextrose should have so readily yielded a strain not attacking it, derived from a strain using it vigorously. The variation in the organism in this case tends in the direction of diminished vegetative activity. Goodman does not state whether on sub-culture without selection the races tended to return to their original characters or not. If in this way he had or had not obtained fair constancy of his races, his results would have been more valuable and complete. This loss of fermenting power so effected, seems

on the face of it at variance with all experience hitherto obtained in the intestinal group. The logarithmic rate of increase of the organisms in the culture would be pulled up after a few hours' growth, after which one would naturally expect that the dextrose present would lead to a constant selection in favour of its fermenters. An increase of fermenting power in these conditions would thus be anticipated rather than a decrease. Goodman takes no cognizance of Neisser's, Massini's and Twort's work and accordingly volunteered no explanation of this occurrence.

De Vries found that in plants, selection from the extremes of the fluctuating variability of a species will not give rise to a new pure race. It will vary the percentage of plants with the special character, to a certain degree, after which further selection seemed of no value, while in the cases of mutation, new and constant races arose immediately.

Of other variations in fermentation power I might add the following :

Hiss (1904) found that the bacillus "Y," a type of *B. dysenteriae*, when cultivated for some time on maltose media, had taken on the power to ferment maltose. This result is confirmed by Lentz and Kutscher (1909). Lentz (1909) also describes a Flexner-strain which in laboratory culture after seven years lost the power to ferment maltose, though still retaining its agglutinating power. Klotz (1906) by passage through an animal, caused an organism to lose a certain fermenting power, but found it was regained by the fourth sub-culture on the particular sugar. The sub-culture lasted only one day. Sub-cultures on ordinary media caused it also to regain the power, only more slowly.

Much old work on variation in fermenting power in the typhoid-coli group is given by Rodet (1894) in his *De la variabilité dans les microbes*, but since he had not the advantage of the agglutination tests one cannot attach much importance to the results. Reliable work of this kind must be done with an organism well defined by many established tests, and evidence of change without these means of identification is almost useless.

Many other functions of bacteria have been made the subject of study in the matter of inheritance, which, since they throw some light on the general aspects of the subject, ought not to be entirely overlooked. Since I have, however, occupied myself mainly with the fermentation characters, I will leave over a consideration of further papers till I endeavour to fit my own findings into the general edifice of modern results on bacterial variation.

## PART I. VARIATION IN THE FERMENTATION CHARACTERS OF THE INTESTINAL ORGANISMS.

*B. typhosus* and *dulcite*. *B. typhosus* is usually stated not to ferment dulcite broth. This is due to the fact that only a short period of observation is undertaken. If the observation is continued for two or three weeks at 37° C., full acidity is usually produced, but occasionally the reaction is slight, even after this period, and careful comparison with a control may be necessary to determine it. Fourteen different strains tested in one experiment showed the first signs of acidity, as indicated by litmus, in from five to fifteen days, with an average of nine days (see Table I).

TABLE I.

The initial reaction of the medium was in each case twelve and a half degrees acid to phenolphthalein. A = full acidity. The various states of partial acidity are indicated by A plus a fraction, the fraction giving an indication of the extent to which the acidity had advanced.

The previously mentioned strains on dulcite broth were sub-cultured after one month at 37° C., when it was found that acidity was produced in from one to four days—average 2½ days—as shown in Table II.

TABLE II.

One month later the same strains were sub-cultured and all showed full acidity in from one to three days; no less than six of them producing sufficient acid to colour the litmus in one day. (See Table III.)

TABLE III.

	"E" 6	"E" 8	"E" 10	Lincoln	"D"	McConn	Gray, F.	"R"	"H"	"W"	King	Guy	Christie
1st day after seeding	A <sub>½</sub>	A	—	A <sub>½</sub>	—	—	—	A	—	—	Did	A	—
2nd do.	A	—	—	A	A	A	—	A	A	not	—	A	—
3rd do.	—	—	A	—	—	—	—	—	—	grow	—	—	—

The first seven strains to produce full acidity in the first generation required in the second generation  $2\frac{5}{7}$  days, while the seven slow ones in the first generation required only  $1\frac{6}{7}$  days in the second generation. This apparent discrepancy arises probably from the injury caused to the organism by the acid produced, which would be greater in the case of the earlier fermenters. It will be seen from the Tables I to III that the first one to produce full acidity in first cultures had died out by the third, and in the second generation caused acidity relatively slowly. As will be seen later, however, at least one other factor influences the period at which sub-culture will give the best result.

Sub-cultures of the first generations of this series of seven were made after 13 days' incubation and these reached full acidity in  $2\frac{1}{4}$  days, i.e., distinctly earlier than when sub-cultured after one month.

The behaviour of *B. typhosus* on neutral-red-dulcite agar was next studied. The typhoid strains of Table I, E 8, E 10, King, Christie and Gray were selected for the purpose as they had all reached the stage of fermenting dulcite broth in one day. For seeding the plates, ordinary broth cultures of the organism, having no previous dulcite inheritance, were used. The results are set forth in Table IV.

The papillae appeared in these strains as early as the third day, and by the fifth day they were acid in reaction. After that time they commenced to fade and took on a dirty yellow colour. The red points that appeared in the centres of the plates on the crowded portions are probably analogous to papillae, as none of the larger colonies took on the acid reaction at any stage of growth.

Quantitative estimation of papillae-bearing colonies in the case of strains Christie, King, and E 10, was made on plates spread from diluted broth cultures. The plates were not at all crowded. On the

sixth day after plating, wide variations were found. Some plates gave as low as 2 per cent. of these colonies and some as high as 50 per cent. The same strain varied within these limits on different plates.

The absolute number of papillae on the colonies possessing them is usually one to three, but occasionally the number rises to as many as 12 by the tenth day, at which time also the proportion of colonies bearing papillae has usually considerably increased.

TABLE IV.

(See Figures 1 and 2, Plate I.)

Plates inoculated, 25/8/09	E 8	King (W)	Christie	Gray, F.	E 10
Observed 26/8	Typical colour- less typhoid colonies	As E 8	As E 8	As E 8	As E 8
29/8	All colonies still without any sign of acid reaction	As E 8	As E 8	As E 8	As E 8
30/8	White papillae appearing on many of larger colo- nies, one to three on each	As E 8	As E 8	As E 8	As E 8
31/8	Some of the papillae are acid in re- action to-day and some acid points are found on crowded por- tions of the plates	As E 8, only the acid pa- pillae are few in number	As E 8, only the acid re- action of pa- pillae is not so marked	As E 8	No red papillae yet
1/9	As yesterday	Acid papillae increasing in number	As E 8	As E 8	A faint sugges- tion of acid reaction ob- tained in some papillae
7/9	The papillae have now faded and are of a buff colour	As E 8	As E 8	As E 8	As E 8

A papilla on a dulcite plate, put through ordinary broth and plated again on neutral-red-dulcite-agar, gave, in the case of "Gray," 50 % red and 50 % white colonies by the fourth day after plating. By the sixth day some of the white colonies showed papillae while others did not.

The ratio of red colonies to white, in the case of E 8, E 10, Christie, and King, was different, but otherwise the plates gave essentially the same results.

The same five strains of *B. typhosus* used in the previous experiment were now grown on dulcrite broth for two days, and on plating again on dulcrite agar gave the result set forth in Table V.

TABLE V.

Plates inoculated, 20/8	Christie	King	Gray, F.	E 8	E 10
Plates observed	Typical colour-less typhoid colonies	As Christie	As Christie	As Christie	As Christie
21/8	Papillae appearing. All colonies colourless	No papillae yet. No acid colonies present	All larger colonies show an acid reaction	As King	As King
23/8	No further change	Papillae appearing	No papillae present	As King	As King
24/8	Papillae are still colourless	Up to seven papillae on one colony	No papillae	No further change	No further change
25/8	Papillae are acid in reaction	As Christie	No papillae. All large colonies smooth and acid in reaction	Papillae acid in reaction	All papillae still colourless
26/8	Papillae faded	Papillae faded	All large colonies have now faded	As Christie	As Christie
7/9					

Result:—The appearances in the case of Christie, King, E 8 and E 10 were similar to those given in Table IV, where the plates were made from normal broths, but all the larger colonies of Gray were fermenters of dulcrite and did not produce papillae, while some small non-fermenting colonies did show them.

After five days' growth on dulcrite broth, three of the above strains were plated on neutral-red-dulcrite-agar with results given in Table VI.

TABLE VI.

Plates inoculated, 23/8	King	Gray, F.	E 10
Observed	No acid colonies present	As King	As King
24/8			
25/8	„ „ „	All larger colonies red	„
26/8	„ „ „	„ „ „	„
27/8	„ „ „	„ „ „	„
1/9	Many acid papillae present in case of larger colonies	No papillae have appeared	..
10/9	Papillae faded	.. .. ..	..

The papillae on the white colonies did not come any earlier after the five days' growth on dulcite broth than they did when plated from ordinary broth. Apparently the variation leaves some of the cells entirely unaffected.

After a period of eight days in dulcite broth, strains "Christie," E 8, and "Gray" had all lost the power to form papillae, but had acquired the power of fermenting the dulcite vigorously. "King" and E 10 showed on the other hand a mixture of smooth red colonies and white papillated ones, the papillae being red. The papillae did not appear till the fifth day.

Red papillae on dulcite plates inoculated into dulcite broth only take three days to produce definite acidity, while the average time of the original strains of the same is eight days. Individuals removed from the smooth part of the colony also required eight days.

If papillated colonies be inoculated into ordinary broth and then plated on dulcite agar, red and white colonies result. One of these red colonies requires only two days to produce acidity in dulcite broth. In this case the non-fermenting elements are entirely avoided.

Normal strains of *B. typhosus*, which by the third generation on duleite broth did not attain the power of fermenting it within 24 hours (Table III), were plated out on neutral red dulcite agar and gave the following result (Table VII).

The observations upon these more slowly varying organisms are set forth in Table VII and may be summarized as follows:

- (1) No red colonies produced by any strain.
- (2) None which are able to produce red papillae.
- (3) Two out of seven not able to produce papillae in the early stages of growth, i.e., within, say, 16 days.
- (4) Five able to produce white papillae.
- (5) One able to produce late papillae.

This series was then plated out from dulcite broth four days old, and II, which had not produced papillae before, showed papillae on the eighth day, probably because of some variation occurring in the broth. Four of the other strains showed a few red colonies.

The effect of the nutrient agar basis of the medium in producing papillae was tested in the following experiment. Eight strains of *B. typhosus* were inoculated on ordinary nutrient agar plates and observed from September 6th to October 14th, 1909, but no sign of papillae-formation was seen. I have never succeeded in getting papillae on single colonies of *B. typhosus* on ordinary nutrient agar, so

that we must look upon the secondary colonies on dulcrite plates as due to the dulcrite, especially since the papillae contain elements capable of fermenting dulcrite to an extraordinary degree. It must however be admitted that one sometimes sees papillae on nutrient agar slopes of *B. typhosus*, but the significance of these has not, so far as I am aware, been determined.

TABLE VII.

Inoculated 6/9	R	Lincoln	McConn	H	Guy	D	E 9
Observed 7/9	Typical colour-less typhoid colonies	As R	As R	As R	As R	As R	As R
8/9	Typical colour-less typhoid colonies	As R	As R	As R	As R	One salmon colony amongst a large number of white ones	As R
10/9	No papillae present. No colonies of acid reaction	As R	As R	As R	As R	Salmon colony faded	As R
13/9	Papillae appearing	All colonies white, and no papillae present	As Lincoln	As Lincoln	As Lincoln	As Lincoln	As Lincoln
15/9	More papillae present, none of them acid however	No papillae present	Papillae have appeared	As Lincoln	As Lincoln	As Lincoln	As Lincoln
16/9	—	Papillae have appeared	—	No papillae present	As Lincoln	Colourless papillae present now	No papillae present
22/9	—	—	—	—	—	—	No papillae present
8/10	—	—	—	—	—	—	Papillae present now

We may, therefore, conclude that the normal *B. typhosus* produces only white colonies on dulcrite agar plates, but some of these may get red papillae and at the same time red points may appear on the crowded parts of the plates, these points being equivalent to papillae. I have never seen a large colony of a normal typhoid strain which was red after 48 hours' growth on dulcrite-neutral-red-agar. The nearest approach was in the case of "D" (see Table VII). In the course of 46 subsequent plates examined after 48 hours' growth at 37° C. I never observed the salmon-pink tinted colony again.

Twort trained on dulcrite broth and his evidence of variation was the production of acidity in broth. He appears to have been unaware that the majority of typhoid strains naturally produce acidity in dulcrite broth. My experience is that every strain will do it without training, though it may be that in the case of some rare strains three or four tubes of the particular strain will be required to demonstrate this fact.

I take it that the strain he trained was peculiarly refractory to dulcrite, and this shows us the need for examining a large number of strains. The normal *B. typhosus*, judging by over 20 strains I examined, will not produce acid colonies on non-crowded plates of dulcrite agar, but one strain in dulcrite broth showed on plating that it contained red elements as early as the second day, and some red colonies were obtained in dulcrite broths from two to ten days old in the case of all strains tested, though not from every single colony.

By the simple expedient of colony selection a typhoid strain fermenting dulcrite in two days may be obtained after only two days' training on dulcrite. See Table V, strain "Gray."

*The sequence of events in the process of variation in the case  
of the "B. typhosus" grown on dulcrite broth.*

This was studied in the following way:

- (1) The total count per c.c. of the broth culture was taken daily.
- (2) The ratio of fermenting to non-fermenting (red to white) elements was determined by plating on neutral-red-dulcrite-agar daily. The plates were observed after 48 hours' growth at 37° C. in each case. They should have 80—100 colonies on the surface and these should be evenly distributed.

Nine such experiments were carried out.

For the purpose of these experiments strain "D" was selected, as it took about 12 days to produce acidity and consequently gave plenty of time for many observations. Occasionally this strain did not become fully acid and it was therefore considered desirable to follow the behaviour of both these varieties.

There are two main types (see Charts I—VII):

In the first type, *i.e.*, the type to go fully acid, the total count is usually 200 to 300 millions per c.c. during the first 7 to 14 days of growth, then it suddenly rises to 600 and to 1000 millions per c.c. Co-incident with this sudden exacerbation of growth the medium becomes



Fig. 1. "*B. typhosus*." Showing secondary colonies as papillae on isodulcrite-agar after five days.



Fig. 2. "*B. typhosus*." Showing secondary colonies as papillae on dulcrite-agar after nine days.



acid. This second maximum may be maintained for one to eight days and is followed by a gradual or sudden fall.

During the first 12 days the organism is using the dulcrite slightly as is evidenced by the fact that the count per c.c. is greater than in peptone water alone during the same time. Daily plating with this strain showed no red colonies during the first week. Reds appeared as a rule after seven and a half days. They remained small in number for a few days until the total count rose suddenly, when simultaneously with this rise the ratio of reds to whites increased.

This late multiplication of organisms is indeed almost entirely due to the production of fermenting individuals as may be seen from the charts.

This course of events suggests some interesting problems. Firstly, what becomes of the whites? Do they multiply and produce reds or not? A number of facts suggest that they do not. In the first place, if whites from a 95 % red plate be put into dulcrite broth, they still take the usual time of the untrained strain to ferment dulcrite, thus suggesting that they have not been impressed by the training. Secondly, if a typhoid strain be grown on dulcrite broth for a few days and then plated on dulcrite agar, the papillae do not appear at all on pink colonies, and on the white colonies they do not usually come substantially earlier than they did originally, suggesting that they have not been impressed. This is seen in some of the tables, *e.g.* Table VI.

In the second type of variation the culture in dulcrite broth becomes only slightly acid and gives persistently a low total count (see Charts VIII and IX).

One of these never exceeded 126 millions per c.c., and the other never 60 millions. The two of this type both showed two rises in total count, the first starting about the 5th day and the second about the 22nd or 23rd day, the second maximum being less than the first. In each case this later maximum was associated with the appearance of reds on plating on dulcrite. The first maximum was also thus associated in one case, but not in the other. After the fall from these maxima the reds disappear to such an extent as to constitute less than 1 % of the total bacterial content, and consequently not to be seen on my plates where I aimed at 80—100 colonies as an ideal number. The persistent low count and the appearance and disappearance of the reds suggest a struggle for existence between the two strains. This varying proportion of reds might partly account for the differences in

time required to produce acidity when sub-cultures are made at various times.

The liability to contamination in these nine experiments was great, so that towards the end of the time during which these variations were taking place in nine dulcrite broths, red and white colonies were selected from the dulcrite plates of eight broths and every one agglutinated with a typhoid serum to 1 in 4000, except one white which went up to 1 in 2000. The test-serum had not a very high titre, viz., 1 in 6000, which was the highest with a number of strains, tested by the microscopic method.

The diagrams suggested, in those cases where full acidity was produced, that the counting of the total number per c.c. is sufficient alone to indicate the variation, and this was shown to be, with certain limitations, reliable. If, for example, a typhoid strain trained to ferment dulcrite in two days, be counted in dulcrite broth on the second day, its count will be about 500 millions per c.c., while a control untrained typhoid will not give more than 200 millions. A one-day dulcrite typhoid will give on dulcrite broth in 24 hours 800 to 1000 millions per c.c. *B. typhosus* gives 116 millions in 24 hours on saccharose peptone water, in lactose about 126 millions, i.e., not substantially different from the count on peptone water, while a lactose fermenting typhoid gives 470 millions per c.c. in 24 hours, when grown on lactose peptone water.

The capacity to ferment a carbohydrate is apparently of value to the organism. In the case of those strains which had attained the power to ferment dulcrite the addition of glucose or dulcrite to the peptone water rendered the medium capable of supporting many times the population possible with peptone water alone. The addition of lactose or saccharose, on the other hand, did not lead to increase of the organisms. Conversely the increase of population in the culture on the addition of a carbohydrate may be taken as evidence of the power to attack it by at least some portion of the population. This effect of a carbohydrate may however be missed unless the counts are made both early and over a considerable period. For example, if the addition of glucose to peptone water, an enormous increase of growth occurs in the case of *B. typhosus*, but if one counts on the second day, the living organisms will be fewer in all concentrations of glucose from 0.5% to 4% than they are in peptone water alone. This is probably due to the high concentration of the acid formed.

Another source of error is that the training is incomplete and the high total does not show itself till the second or third day. This would be avoided by further training or slightly later counting.

*Permanency of the new character.*

In respect of the permanency of the dulcrite fermenting character I may adduce the following facts: When "Gray" and "Christie" (see Tables I, II and III) had reached the stage of fermenting dulcrite broth in one night, red colonies from dulcrite plates of these strains were inoculated into peptone water, and sub-cultured 25 times from August 28th, 1909, to January 5th, 1910. It was then found that 100% of the colonies on dulcrite plates were still all intensely red, so that apparently the character had not commenced to recede. Two other one-day dulcrite typhoids, carried concurrently with the above to the tenth generation on peptone water, also gave 100% of reds on dulcrite plates.

We have seen that in the training of strain "D" to ferment dulcrite the plating showed usually an irregularly increasing ratio of reds to whites if the history in dulcrite broth was followed. Now when the ratio was 14%, reds, 90% reds, and 100% reds, colonies were picked off to test the permanency of the character at these different stages.

Three red colonies at each stage were inoculated into peptone water and sub-cultured in it ten times between October 29th and December 26th. It was then found that they were all losing their character, i.e., were giving rise to white colonies as well as reds. In the first generation all the progeny were red, in the second, one of the 14% reds gave only 31 reds out of a total of 45. The other eight of the series gave 100% of reds. By the fifth generation all the 14% series and one of the 90% showed whites, and after the seventh sub-culture all the series showed signs of losing the character.

*Variations in individual strains.*

It was next determined to further investigate the great differences observed as to degree and time of acidity when *B. typhosus* is grown in dulcrite broth. These were thought to depend on the strain used, but this was found to be only a subsidiary factor, as Table VIII shows.

Twenty different colonies from a MacConkey plate of pure typhoid, freshly isolated from faeces, were inoculated into dulcrite broth, and the time of acidity noted (see Table VIII).

TABLE VIII.

21/10	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
7th day after seeding	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
9th do.	—	—	—	—	—	—	—	—	—	—	?	—	—	—	—	—	—	—	
10th do.	—	—	—	?	—	—	—	—	—	—	?	—	—	—	—	—	—	—	
11th do.	—	?	?	"	—	—	?	—	A	"	?	A $\frac{1}{4}$	—	—	—	?	?	?	
12th do.	—	A $\frac{1}{2}$	A $\frac{1}{2}$	A $\frac{1}{4}$	—	—	"	—	—	"	A $\frac{1}{4}$	A $\frac{3}{4}$	—	—	—	"	"	"	
13th do.	—	A	"	A $\frac{1}{2}$	—	—	A $\frac{1}{2}$	—	—	"	A	A	—	—	—	"	"	"	
14th do.	—	—	"	"	—	A	A $\frac{1}{2}$	?	—	"	—	—	?	?	?	"	"	"	
15th do.	—	—	"	"	A $\frac{1}{2}$	—	—	"	"	—	—	"	"	—	"	"	"	"	
17th do.	—	—	"	"	—	—	"	A $\frac{1}{4}$	—	"	—	—	"	"	—	A $\frac{1}{2}$	"		
18th do.	—	—	"	A $\frac{3}{4}$	A	—	—	"	—	A $\frac{1}{2}$	—	—	"	"	—	A $\frac{1}{4}$	A $\frac{3}{4}$	A $\frac{1}{2}$	
19th do.	—	—	"	—	—	—	"	"	—	—	—	—	"	"	—	"	A	A	
21st do.	—	—	"	"	—	A $\frac{1}{2}$	—	A $\frac{1}{2}$	—	"	—	—	A $\frac{1}{2}$	—	A $\frac{1}{2}$	—	"	—	
22nd do.	—	—	"	"	—	"	—	"	—	"	—	—	"	"	—	"	—	—	
24th do.	—	—	"	"	—	"	—	"	—	"	—	A	"	A	—	"	—	—	
25th do.	—	—	"	"	—	"	—	"	—	"	—	—	"	—	—	A $\frac{1}{2}$	—	—	
27th do.	A $\frac{1}{4}$	—	"	"	—	"	—	"	—	—	—	—	—	—	—	—	—	—	
32nd do.	A $\frac{1}{2}$	—	A	A	—	A	—	A	A	—	A $\frac{1}{2}$	—	—	A $\frac{1}{2}$	—	A $\frac{1}{4}$	—	—	
34th do.	A	—	—	—	—	—	—	—	—	A	—	—	"	"	A	—	—	—	
37th do.	—	—	—	—	—	—	—	—	—	—	—	—	A	—	"	—	—	—	

The experiment was repeated with a laboratory strain of *B. typhosus* and 21 tubes were inoculated all from a single colony. The earliest took seven days to produce full acidity and a number never went fully acid, although by the 23rd day acidity appeared in all the tubes. In some, however, it remained slight and indifferent.

In reference to strain, it may be said that if a particular strain usually takes a shorter time than another in producing acidity, it will vary within narrower limits, e.g., strain "Wright" took (see Table I) only five days to produce acidity, and on testing four tubes of it I found they all produced full acidity on the same day, with insignificant variations of degree of colour change. "Christie," "King," "E 10," and "E 8" had three tubes of each tested and showed less uniformity than "Wright," but greater than the strains having a longer usual time (see the following Table IX).

The group of tubes of each strain was inoculated from the same colony. Up to the fifth day they showed no sign of acidity, except Wright.

It was next thought that the size of the seeding might be an important factor. Assuming that the first maximum with *B. typhosus* in dulseite broth is 200 millions per c.c., then if 100 organisms are used

as a seeding, the number of generations needed to increase this number to 200 millions will be about 20. If, on the other hand, five millions be inoculated, then very few generations, viz., about seven, will produce the same number. Now, other things being equal, the amount of

TABLE IX.

	Christie			King			E 10			E 8			Wright			
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	4
6th day after seeding	A $\frac{1}{2}$	—	A $\frac{1}{2}$	A $\frac{1}{2}$	—	A $\frac{1}{2}$	A $\frac{3}{4}$	A $\frac{3}{4}$	A	A						
7th do.	A $\frac{1}{2}$	A	A $\frac{1}{2}$	A $\frac{1}{2}$	A $\frac{1}{2}$	—	A $\frac{1}{2}$	A	A	—	—					
9th do.	A	„	„	A	„	„	—	A $\frac{3}{4}$	A $\frac{3}{4}$	„	—	„	—	—	—	—
10th do.	—	„	„	—	“	“	—	“	“	“	—	A	—	—	—	—
13th do.	—	„	„	—	A	„	—	A	A	„	—	—	—	—	—	—
16th do.	—	„	„	—	—	“	—	—	—	A $\frac{3}{4}$	?	—	—	—	—	—
22nd do.	—	A	A	—	—	A	—	—	—	A	A $\frac{1}{2}$	—	—	—	—	—

selection possible will be proportional to the number of generations, and therefore it seemed reasonable to suppose that the smallest inoculation would cause the earliest acidity. This view led to the following experiment:

Twelve dulcite broths were divided into four groups of three each, and were seeded at the same time from the same culture as follows:

First group with 98 organisms each.

Second group with 19,600 organisms each.

Third group with 1,960,000 organisms each.

Fourth group with 5,880,000 organisms each.

The time of acidity is shown in the following table (Table X).

One of the first group shows a very early acidity. On the other hand the last to get to the stage of "A $\frac{3}{4}$ " was a small seeding, so that, speaking in rough general terms, a small seeding will in a series give more irregular results, but not of necessity earlier acidity. If a series is put up with a large seeding, still great irregularity in results is found, because the normal typhoid cells of the same strain differ greatly in their relationship to dulcite. It might be possible with still larger seedings to get uniformity in the time required to produce acidity, but of this I have no positive evidence.

How are we going to class the dulcite fermenting power which *B. typhosus* takes on in this way? Nearly all its strains can be shown capable of producing papillae on dulcite. One-third of them can produce papillae which are red in character on the neutral red medium,

and in this they correspond with Massini's mutation; on the other hand the new strain in its early stages produces many atavists, i.e., non-fermenting progeny which, apparently, further growth on dulcite removes altogether. This is quite opposed to mutation. Moreover, the fact that long training on dulcite removes the atavistic tendency shows that a new race can be obtained of great stability by long continued selection, a result de Vries could not get with the plants he experimented with.

TABLE X.

	1st Group 98 Organisms as Seeding			2nd Group 19,600 Organisms as Seeding			3rd Group 1,360,000 Organisms as Seeding			4th Group 5,880,000 Organisms as Seeding		
	1	2	3	4	5	6	7	8	9	10	11	12
5th day after seeding	—	—	—	—	—	—	—	—	—	—	—	—
6th do.	—	—	A	—	—	—	—	—	—	—	—	—
12th do.	A½	—	—	—	—	—	—	—	—	—	—	—
13th do.	"	—	—	A½	A½	—	A½	—	A½	A½	—	A½
15th do.	"	—	—	"	"	—	"	—	"	A½	—	"
16th do.	"	—	—	"	"	A½	A½	—	A½	"	—	"
19th do.	"	A½	—	A½	A½	"	"	A½	"	"	A½	A½
21st do.	"	"	—	"	A½	A½	"	A½	"	"	"	"
22nd do.	"	"	—	"	"	"	"	"	"	A	A½	"
23rd do.	A½	"	—	"	"	"	"	A½	"	—	A	"
27th do.	A	"	—	A½	"	A	"	A	"	—	—	"
30th do.	—	"	—	"	"	—	A½	—	A½	—	—	"
32nd do.	—	A½	—	"	"	—	"	—	"	—	—	A½
35th do.	—	A½	—	"	"	—	"	—	"	—	—	"

## PART II. THE FERMENTATION OF LACTOSE BY THE *B. TYPHOSUS*.

Dr Twort was kind enough to supply me with a typhoid strain which he had trained to ferment lactose, and I examined the same fully, as also its progenitor. Successive sub-cultures on a lactose medium for a period of two years had been required to develop this strain. It showed the following characters: It was an actively motile bacillus and had the biological characters of the normal *B. typhosus* on the ordinary media, except that on broth it produced a marked scum. This latter property will be referred to later.

It fermented glucose, mannite and lactose overnight, without gas formation. Saccharose was not fermented while sorbite was attacked in an irregular fashion. Out of 10 tubes of sorbite only one showed acidity by the ninth day at 37° C. The others showed acidity

later. This irregularity in the sorbite character belonged to the original normal strain which I had an opportunity of examining, and was in no way due to the lactose variation. The organism did not produce indol.

For the study of its agglutination characters, broth cultures could not be used, as in spite of repeated trial they showed a great tendency to spontaneous agglutination. Agar slopes of 24 hours' growth, however, gave no trouble in this way (*vide* Table XI).

TABLE XI.  
*Macroscopic Agglutination Tests.*

			Twort's Lactose Typhoid Strain	G.—A Control Typhoid Strain	
Typhoid serum (Wilson)	1 in	20	+	+	+
		100	+	+	+
Observations made after $1\frac{1}{4}$ hrs. and $2\frac{1}{2}$ hrs.		200	+	-	+
(No change between the two observations)		400	+	+	+
		800	+	+	+
		1,600	+	-	+
		3,200	+	+	+
		6,400	+	+	+
Normal horse serum	1 in	20	+	+	+
		100	+	+	
Observations made after $1\frac{1}{4}$ hrs. and $2\frac{1}{2}$ hrs.		200	+		
(No change between the two observations)		400	-		
		800	-		
		1,600	-		
		3,200	-		
		6,400	-		
Normal saline solution and typhoid emulsion in 2 hrs.			No agglutination		
Typhoid serum (Wilson)	1 in	20	+	+	+
Observation after 24 hrs.		100	+	+	+
		200	+	+	+
		400	+	+	+
		800	+	+	+
		1,600	+	+	+
		3,200	+	+	+
		6,400	+	+	+
Normal serum	1 in	20	+	+	+
Observation after 24 hrs.		100	+	+	+
		200	+		
		400	-		
		800	-		
		1,600	-		
		3,200	-		
		6,400	-		

*General result.* Twort's trained organism differs only from normal *B. typhosus* by the formation of a scum on broth and by the fact that instead of fermenting sorbite in one day it requires about 10 days, and in some cases does not do it at all definitely in the first culture. The agglutination tests, however, together with all the other fermentation tests except lactose, and also its morphology and motility, show it to be a genuine typhoid bacillus. It is much to be regretted that the organism had these two striking deviations from the type, as it is difficult to say whether they may not have been potent in assisting the training or even of making it possible. Konradi (1904) has stated that *B. typhosus*, after growth for a long period in water, can take on this power of scum formation, so that its presence is not inexorably opposed to the organism being *B. typhosus*. Apart from this statement of Konradi, however, I can find no reference in the literature to the formation of a scum on nutrient broth by the *B. typhosus*.

To ascertain whether the organism was a pure strain in reference to the lactose-fermenting character, it was plated out on MacConkey's medium, *i.e.* neutral red lactose bile salt agar, and incubated at 37° C. On the second day after plating a mixture of red, salmon-pink and white colonies was present. Up to the sixth day the salmon-pink colonies tended to become a little deeper in colour but subsequently showed no further change. The difference in colour is not entirely a question of crowding as some of the white colonies were standing alone in similar positions to red colonies. This was repeated on two plates when the reds amounted to 50 per cent. of the total, the non-crowded portions only being counted.

The plating was also undertaken on lactose agar without bile salt. The only difference that could be detected was that the percentage of reds in the earlier days was less than on MacConkey's medium. The absolute number of reds continued to increase until the twelfth day.

The plating was also undertaken from lactose-peptone-water a few days old and fully acid, when it was found that the percentage of reds on the MacConkey plates on the sixth day reached 95 per cent.

Plates were also made on the same medium from the scum on the broth, and it was found that the reds were fewer and later in appearing than those taken from the broth itself. The difference was, however, not very marked.

As the above facts pointed to great instability in respect of lactose-fermenting power, five red colonies and five white were picked off and put on to agar slopes for further examination.

The lactose-fermenting and non-fermenting strains, which for the sake of brevity I shall call the red and white respectively, agreed in all their characters, except in the power of fermenting lactose. All the five red strains gave acidity in lactose-peptone-water overnight, while only two of the white strains became acid, although they were observed for a month, viz., one on the seventh and one on the ninth day. No indol was produced by any of the strains. Agglutination and absorption tests gave the following result:

TABLE XII.

*Typhoid Serum (Wilson, Titre 1 in 12,000). Macroscopic Method.*

Typhoid serum		Red strain after 2 hours	White strain after 2 hours
1 in	50	+	-
100		+	+
200		+	+
400		+	+
800		+	+
1,600		+	+
3,200		+	+
6,400		+	+
Normal horse serum			
1 in	50	+	+
100		..	+
200		-	-

The above typhoid serum was absorbed with the white strain by Castellani's method, and it was found to absorb all the agglutinin for itself, the red strain, and also a stock typhoid strain, in dilutions from 1 in 150 to 1 in 5,000 (Table XIII).

TABLE XIII.

*Typhoid Serum (Wilson) absorbed with white strain. Macroscopic Method.  
Observed after 2 hours.*

Dil. of Serum after being absorbed	White strain	Red strain	Strain L
1 in	50	+	+
150	-	-	-
500	-	-	-
1,000	-	-	-
5,000	-	-	-

A partial absorption by "H," another known strain of typhoid, gave the following results with the same serum (Table XIV).

TABLE XIV.

*Typhoid Serum absorbed with "H."*

Serum	"H"	"G"	Red Twort
1 in 100	+	As "H"	+
200	+	"	+
400	+	"	+
800	+	"	+
1,600	+	"	-
3,200	-	"	-

The titre of the serum with "H" was 1 in 12,800.

Another partial absorption of the same serum with "H" gave maximum agglutination with "H" and the red strain organisms at 1 in 100.

That these strains, therefore, both red and white, were identical except in respect of the lactose-character was manifest. So far as agglutination tests went, they were both genuine typhoid organisms.

*Investigation of the progeny of the red and white colonies.*

Red, salmon-pink and white colonies were plated out from broth on MacConkey's medium in order to study the percentage of the particular colonies in the progeny. Botanists find that the selection of races is best furthered, not by selecting divergent individuals, but rather by selecting individuals, which in their progeny show an average high development of the divergent character. This is expressed by the percentage which conforms to some arbitrary standard, the "hereditary percentage." I attempted the same method here and found that like colonies gave very different results on plating. The results stated are obtained by taking two separate sets together.

The red strains gave on the third day 96% of reds and this percentage did not increase. In none of the colonies was the redness so intense as when the plating took place from lactose-peptone-water a few days old.

The salmon-pink colonies on plating gave a mixture of reds and pinks on the one hand and whites on the other. On the fifth day the fermenting colonies were in excess.

The white colonies on plating gave only whites though observed for fourteen days. Each of the above observations was made from a consideration of two sets of plates.

The position would appear to be the following: the red strain is constantly throwing off atavists of white and red; the salmon-pink produces red, salmon and white; but the whites show no tendency to produce progeny of the new variety.

Rapid sub-culture of the red strain on peptone water without lactose was next undertaken, the sub-cultures being made every three days, in order to see if the lactose-fermenting property would be rapidly lost. Two series of cultures were so treated, derived from different red colonies. The fifth sub-culture plated out on lactose without the bile salt showed after four days, no red colonies in either set of plates. The same sub-cultures on MacConkey's medium showed no red colonies in one set of plates, but on the set from the second culture 6% of red colonies appeared.

The tenth sub-culture was plated out on MacConkey's medium and on the fourth day no red colonies were present in either set. On the fifth day, however, a few salmon-pink colonies appeared in the crowded portion of one plate, but none of the large independent colonies showed the slightest suspicion of acidity. The pink central colonies took three days to produce acidity in lactose-peptone-water. Some whites tested produced no acidity in ten days.

The two series of red strains were plated out again on MacConkey's medium after the twentieth sub-culture on peptone water. They were observed daily for 12 days and during that time did not show a single red or pink colony, either in the crowded or scattered portions of the plates, though some of the colonies were half an inch in diameter by the fifth day.

The sixth sub-culture on peptone water was inoculated into litmus-lactose broth (two tubes) and produced acidity, one on the fourth day and the other on the fifth day. The fifteenth sub-cultures were inoculated into four tubes of litmus-lactose-peptone water. One became acid on the seventh day, two on the eighth day, and one on the eleventh.

It is evident, therefore, that though the organism was capable of fermenting lactose it was not in a position of stable equilibrium and was always reverting towards its normal condition.

It seems permissible to say that the greater the number of generations passed through in peptone water, *i.e.*, the longer the period since

the selective process has been in abeyance, the nearer will the average of the progeny approach the normal.

This lactose-fermenting typhoid can scarcely be dignified with the title of a new variety on account of this constant tendency to reversion. This same tendency was present in the early stage of the dulcite training, however, but, as we have seen, a prolongation of the training produced a stable and pure race. The stable dulcite position can be sometimes attained in two to three months' training, while two years' training in the case of lactose is required to bring it to the early stage, *i.e.*, of producing fermenting progeny always tending to throw off atavists. The early stage in dulcite training is attained in two to ten days, and if the analogy could be permissibly carried further, it seems only natural to believe that a stable and perfectly constant lactose-fermenting race of typhoid, proving itself to be such after 20 or 30 sub-cultures on peptone water, would require very many years of this continuous training-process for its selection.

This process might be quickened by the systematic use of colony selection as the sub-cultures were made.

*Author's attempts to produce a lactose-fermenting strain  
of the "B. typhosus."*

The training of *B. typhosus* to ferment lactose was next undertaken. For this purpose, many normal strains were used and also the white atavists of Twort's lactose-fermenting typhoid strain which we have just been studying.

The training was carried out on lactose agar slopes with neutral-red and also on litmus-lactose-peptone water. The solid medium was necessary in order to ascertain if papillae were formed as in the case of mutation.

Table XV gives the results of training *B. typhosus* on neutral red lactose agar in order to select out a lactose-fermenting strain. White atavists of Twort's strain were used and also other ordinary strains of *B. typhosus*.

The three separate strains of white atavists of the lactose-fermenting typhoid strain during retraining on a solid medium containing lactose, produced papillae, small at first, becoming on further sub-culture large and red, and followed on still further sub-culture by small papillae which ultimately coalesced to form a broad thick deep eoli-red streak. All the three strains agreed in the formation of large red papillae, but the

TABLE XV.

To show the history of training various typhoid strains on neutral-red-lactose-tryptar slopes.

Organisms and results of examination.

Dates of sub-culture		White Atavists of Twort's Lactose typhoid			G. A Laboratory strain of Normal <i>B. typhosus</i>			L. A Second strain of Normal <i>B. typhosus</i>		
		(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)
5th March, 1909	"	—	—	—	—	—	—	—	—	—
15th " "	"	—	—	—	—	—	—	—	—	—
15th April "	"	Shows fairly large papillæ of neutral reaction	<i>Large acid papillæ develop</i>	Smooth growth	Developed tiny neutral papillæ	—	—	—	—	—
3rd May "	"	—	—	—	—	—	—	—	—	—
24th May	"	<i>Large acid papillæ develop</i>	A thick dirty reddish streak appears without papillæ	"	"	Tiny papillæ of neutral reaction developed	—	—	—	—
22nd June	"	Small papillæ developed of similar colour to rest of growth	Small papillæ appear	"	—	—	—	—	—	—
18th August	"	"	"	Small neutral papillæ appear by the fifth day	Developed tiny papillæ of neutral reaction	—	—	—	—	—
20th Sept.	"	Growth remained smooth, the acid reaction fading slowly	Small papillæ appeared by the eleventh day	<i>Large coarse papillæ of acid reaction</i>	Developed tiny neutral papillæ	—	—	—	—	—
10th October	"	"	Growth remained smooth	Growth remained smooth	Developed tiny papillæ	—	—	—	—	—
11th Nov.	"	"	"	"	Developed tiny neutral papillæ	—	—	—	—	—
10th Dec.	"	"	"	"	Developed tiny neutral papillæ	—	—	—	—	—

The cultures which are not marked in showed during their whole growth no papillæ formation.

time of formation was not always the same, though the training ran concurrently in all. This shows that there is considerable variety amongst the atavists.

The training of normal typhoid strains of the same series shows in the first year a tendency to the appearance of small papillae in from 6 to 20 days after the sub-culture is made. These may appear repeatedly on the same strain; they have the same colour as the rest of the growth, and are very small, quite unlike the large papillae observed in the later stages of training of the before-mentioned lactose-atavists.

The significance of these papillae is difficult to determine. They may be due to the lactose in which a selection occurs, in favour of organisms using it rather better than their neighbours though unable to produce an acid reaction in the medium, but it is also possible that they may indicate a selection going on in favour of strains which can use the peptone basis of the medium more fully. I incline to the former view. It seems the explanation of the large papillae with acid reaction, as they are accompanied by such an augmentation in their fermenting power on lactose. In the case of the small papillae which frequently recur, I believe them to be also due to the lactose, since papillae on nutrient agar without sugar are not frequent and the papillae have a somewhat different appearance. On nutrient agar they are slightly larger and not so sharply defined from the growth on which they occur.

The three white atavists of Twort's lactose-typhoid strain that did not ferment lactose were sub-cultured into litmus-lactose-peptone-water after one month, and then again after a second month. On the sixth day after this sub-culture, white strain I became fully acid. On the eighteenth day after sub-culture, strain II showed distinct but not complete acidity, while the third remained unaffected until the twentieth day, when it showed slight acidity. The controls of ordinary *B. typhosus*, three of "G" and three of "L," were unaffected. Sub-culture was again undertaken after one month and all showed slight acidity two days after the sub-culture. On the third day they were all fully acid. Sub-culture was again undertaken after a month, and on the second day after, one strain was fully acid, the other two half-way turned. The following day they were all fully acid. Sub-cultured again after an interval of seven weeks, one became acid on the first day. The last sub-culture was made at a long interval, from unavoidable causes. At a time when the fourth sub-cultures showed acidity in all the tubes, viz., on the fifth day, it was sub-cultured to see if it was necessary to

have these long intervals between the sub-cultures. They all showed slight acidity on the second day again, but no sign of it on the first. The fourth sub-cultures plated out on MacConkey's medium, five days after their inoculation, showed no sign of acidity until the second day, when white strain I showed a few pale salmon tinted colonies, the other two strains, white only. On the fourth day, I and II both showed salmon colonies; III showed a very few red ones. They showed no subsequent change.

Three strains of "G" and three of "L" have been growing on litmus-lactose-peptone-water since February (1909), being sub-cultured each month. At the end of a fortnight they only give an indefinite acid reaction. Compared with a control they apparently show slight acidity, but it is not definite. I have also trained Twort's normal typhoid strain from which he developed his lactose fermenter, since March 31, 1909, up to date (November, 1910), by means of fortnightly sub-culture in the case of three series, and monthly sub-culture in another three series, and while they both at the end of a fortnight appear identical and very slightly acid, the training appears to have been so far of but little value.

On June 3, 1909, I put a further series of 12 different typhoid strains on lactose-peptone-water, some fresh from the body and some old. They have been sub-cultured every month up to date. They are all difficult to train as far as one can at present judge. I also attempted the training during four months (January—April, 1910) of various dulcrite-fermenting typhoids, but with no greater success. So far as I can judge, therefore, normal typhoid strains cannot acquire this character by this method of training in one year. Atavists of a trained lactose-fermenting typhoid can be fairly easily retrained.

The training of typhoid to ferment lactose was also undertaken by more rapid sub-culture, viz., every four or five days, and this was continued in the case of "G" (two strains) for 42 generations, and in two strains of another typhoid organism for 38 generations, without any more effect than was produced in the slow series of sub-cultures carried on during the same time. Plating out on MacConkey's medium at any stage of the above training failed to produce red colonies.

A series after five monthly sub-cultures on lactose-peptone-water was then sub-cultured every week for 13 generations and produced nothing more than slight indefinite late acidity.

## PART III. TYPHOID AND ISODULCITE.

Reiner Müller has described the formation of papillae on isodulcite by the *B. typhosus* and found that they might be composed either of fermenters or non-fermenters of isodulcite, that is to say, they might be red or not, on media with a suitable indicator. I have tested 20 strains of *B. typhosus* from different sources on neutral-red-isodulcite-agar and have found a well-defined papillae-formation appear with every strain, in from three to five days. The papillae might reach 30 or even more in number and were present in all the large discrete colonies. In no case, however, did I find the papillae red. All these strains were inoculated into ordinary broth from a single papilla and replated on isodulcite-neutral-red-agar with the result that I obtained in nearly every case two sorts of colonies, the first showing quite large naked-eye papillae, the second smooth to the naked eye and of denser growth, but in no single instance red in colour. The examination of these smooth colonies with a lens frequently reveals very tiny papillae, but not at all similar to the original ones.

Dulcrite- and lactose-trained typhoids give these papillae on isodulcite.

As Reiner Müller occasionally obtained acid papillae, which when sub-cultured gave permanently acid strains, I observed the time taken by a few strains to produce acidity in isodulcite broth. The lactose-typhoid took 19 days, but when sub-cultured after 21 days it became fully acid in 11 days and a second sub-culture after similar treatment produced full acidity in two days.

A dulcrite-fermenting typhoid strain required in the first isodulcite-broth culture 19 days, on first sub-culture six days, and on the second two days.

A normal typhoid strain "D" took only seven days in the first broth-culture, four in the second, and two in the third.

Another stock typhoid strain was very obstinate, and after the third monthly sub-culture it took 21 days to produce full acidity; after the fifth sub-culture it became fully acid in three days.

Two other strains of typhoid occupied an intermediate position in respect of time taken to acquire the fermenting power on this particular carbohydrate.

The testing of the constancy of this character (*i.e.* absence of papillae-forming power) to obtain evidence of mutation is only of value in the

presence of a positive result. To carry out the test a number (four) of typhoid strains varied towards isodulcite so that they grew on it luxuriantly without being able to produce papillae, were put through 13 generations of peptone water during three months and on replating on isodulcite not one of them produced papillae again. If in this case the evidence of bacterial mutation had not been positive it would have been difficult to draw any conclusion, as the *B. typhosus* occasionally produces papillae on ordinary nutrient agar for some cause as yet undefined.

*Subvariants.* The first indication of the acquirement of the dulcite character may be the appearance of salmon-pink colonies, the centres not being markedly deeper in colour than the rest of the colony, and this probably must be looked upon as a subvariant. I have sometimes found dulcite broth almost entirely composed of these elements. More frequently, however, the new character is shown by a small deeply coloured spot in the centre of an otherwise white colony. In this connection I might mention that plating out from broths in which mutation is occurring, at frequent short intervals, e.g. every hour, and observing the character of the cells, does not appear to have been done, so that the assumption that bacterial mutation exists (without the occurrence of subvariants in the process) as in botanical mutation is not yet absolutely proved.

#### SUMMARY OF RESULTS.

(1) *B. typhosus* ferments dulcite-peptone-water in about 10 days. By sub-culture this is readily shortened to one day. It produces non-fermenting colonies on dulcite-agar plates. The colonies show after five days and upwards, secondary colonies as papillae, which may or may not have an acid reaction.

Growth of *B. typhosus* on dulcite media readily gives rise to bacilli forming, from the commencement, acid colonies on dulcite plates. The dulcite character has a tendency to atavism in the early stage which further growth on dulcite removes.

(2) *B. typhosus* can be trained to ferment lactose, only with very great difficulty. Definite and conclusive results have been obtained by me only with the white atavists of Twort's trained lactose strain. Other strains (20) have not yielded to such training methods, although many of them have been trained for over a year. The lactose character

when acquired is very unstable, and is soon lost when growth takes place in a lactose-free medium. A fermenting colony on replating gives rise to a mixture of fermenting and non-fermenting colonies.

(3) All strains of *B. typhosus* which I have examined show the secondary colonies on isodulcite described by Reiner Müller. Growth on isodulcite yields directly a strain without this papillae-forming power. So-called "acid" papillae as described by Reiner Müller have not been observed in the strains (20) investigated by me. This new strain does not regain the power to form isodulcite papillae by frequent sub-culture apart from isodulcite. Apparently the acquired character has arisen by mutation. Fermentation of isodulcite requires a considerably longer process of training.

(4) The above three characters vary in permanency. The permanency varies inversely as the time required for the selection or training.

(5) Papillae-formation arising on carbohydrate media on colonies of members of the typhoid-coli group may indicate variation by slow degrees just as well as by mutation. I have observed it in normal typhoid varying towards dulcite and lactose as well as isodulcite. In the case of Twort's strain which fermented sorbite slowly, I observed papillae-formation on sorbite-agar.

Formation of papillae simply indicates apparently that the variation only affects very few individuals of the colony to a great extent, and does not guarantee permanency in the character.

If the papillae arise early and without sub-culture, the evidence adduced so far suggests that the character will have considerable permanency. In the case of dulcite the results show that the formation of papillae on certain carbohydrate media cannot, as Reiner Müller stated, be regarded as definitely specific for the variety of micro-organism exhibiting them.

The variations detailed in the above communication do not in our view invalidate the sugar tests as aids to differentiation, since these chemical functions are subject to variation probably to no greater extent than other functions of bacteria. Further, the ability of an organism to vary in any particular direction may be of considerable value in differential diagnosis.

This instability makes it imperative to have as large a number of differentiating characters as possible for every species, and also to take care in laboratory culture that we do not grow bacteria under conditions likely to alter their characters. It would be inadvisable for example

to propagate the *B. typhosus* from a duleite culture a few days old if one desired it to conform to the normal typhoid type in respect of duleite.

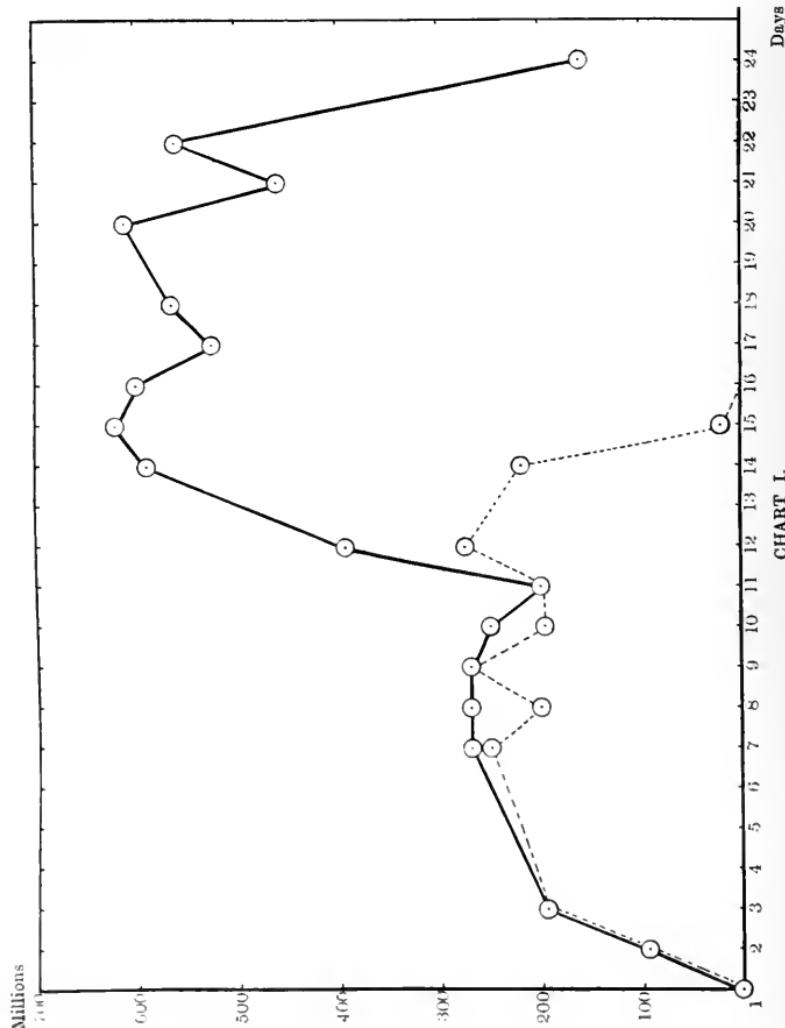
In sending this paper to the Press I would like to acknowledge my indebtedness to Dr Martin, who has supervised my work on behalf of the British Medical Association, and to Dr Ledingham, who suggested the work, from both of whom I have received much valuable help and criticism. Many other colleagues at the Lister Institute have helped me from time to time in points of difficulty, and I take this opportunity of thanking them.

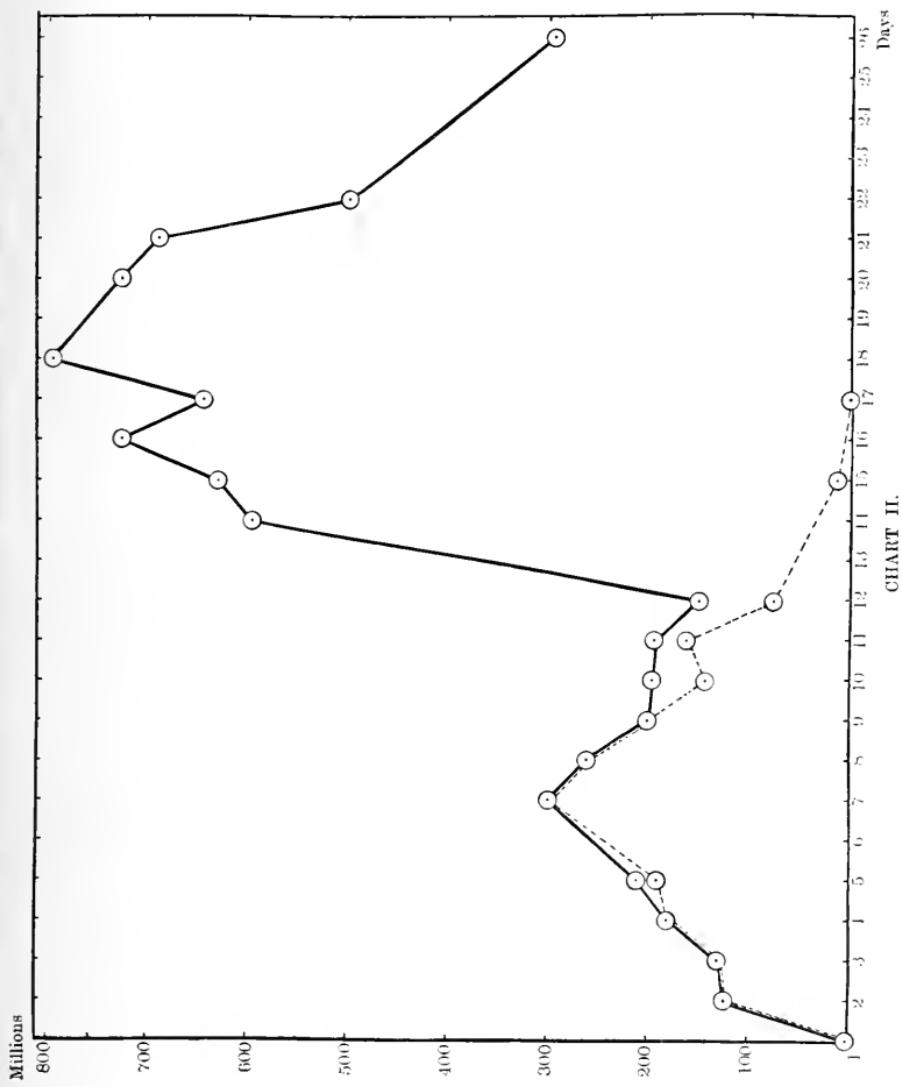
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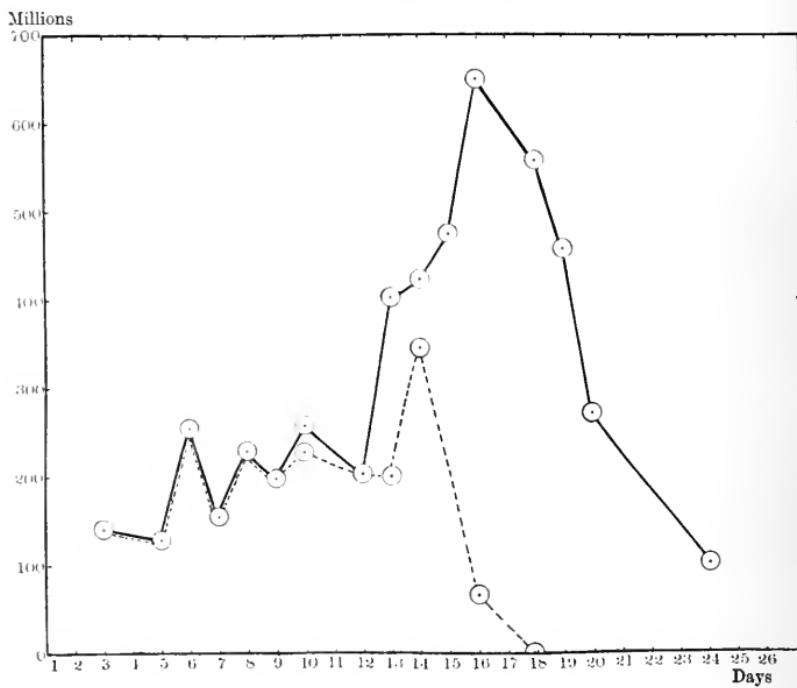
*Bacterial Variation*

CHART III.

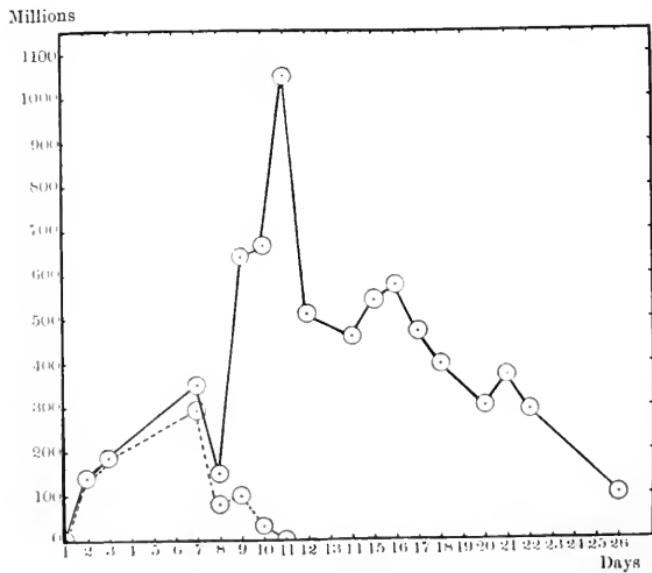


CHART IV.

Millions

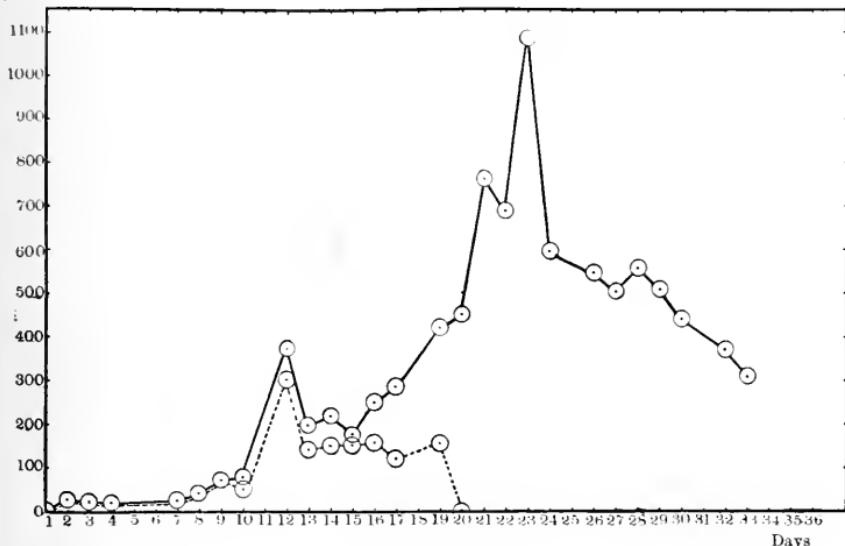


CHART V.

Millions

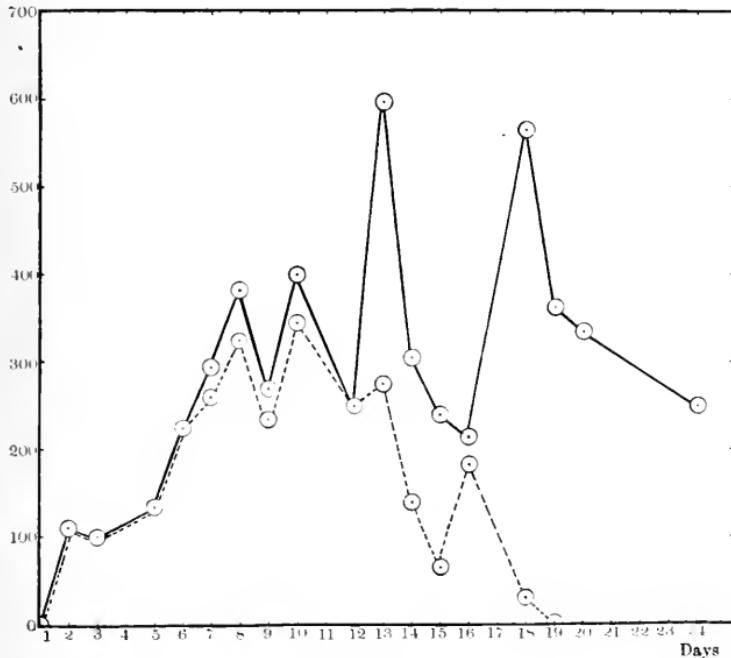


CHART VI.

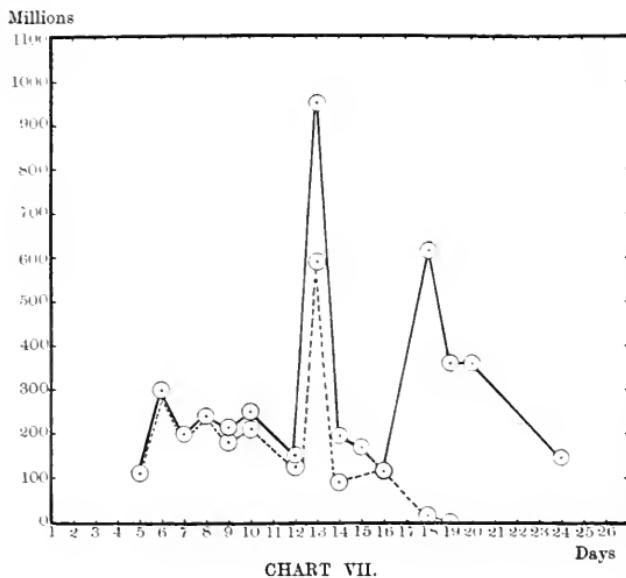


CHART VII.

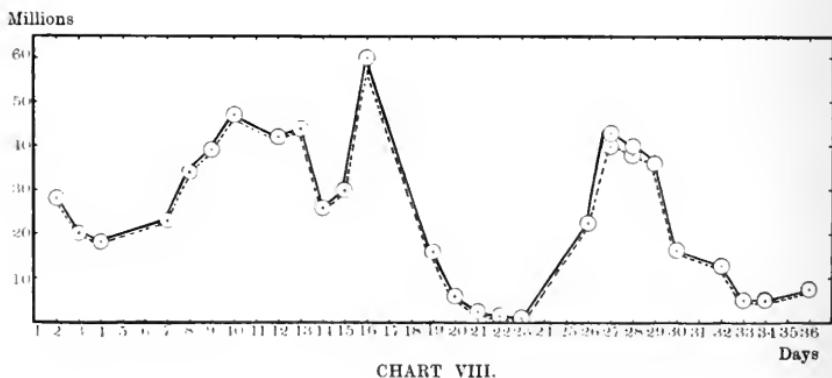
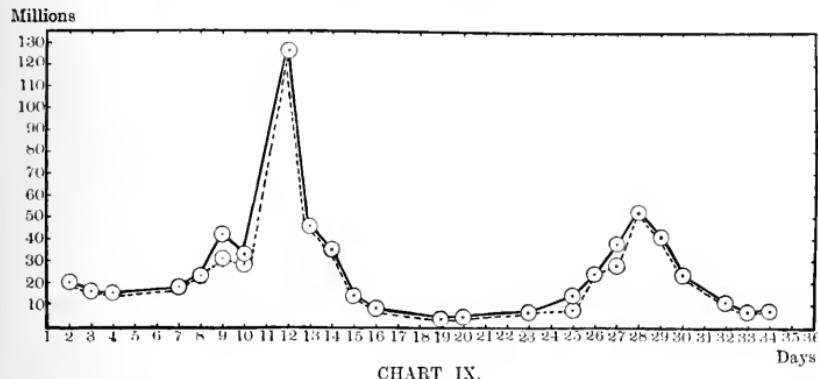


CHART VIII.



### EXPLANATION OF CHARTS.

*Charts I to VII.* The upper line shows the total count in dulcite broth from day to day during the process of variation expressed as millions per c.c.

The dotted line indicates the numbers of non-fermenting elements in the broth obtained by considering the total count and the ratio of red to white colonies on neutral-red-dulcite agar plates.

This gives the history of a dulcite broth becoming fully acid in about a fortnight.

*Chart V* shows that the dulcite broth had a very low total count per c.c. for ten days and thus appeared to be of the second type. At this stage, however, it changed suddenly and became like the first type almost in all respects.

*Charts VI and VII* show a sudden increase in numbers; coincident with this increase the acidity rises and the subsequent rapid death is presumably due to this cause. A few days later a similar sudden increase and diminution occurs. Had this happened only in one culture, it might have been due to clumping, but the identity of the two charts makes me believe such an explanation is insufficient.

It seems more likely that the first selection, under the influence of which a dulcite-fermenting strain was produced, led inevitably to increased acidity which occasioned the death of the majority of the individuals, leaving only those more resistant to this adverse influence. Subsequently a new stock was bred from these, to be in their turn destroyed by a further rise in acidity.

I have found for example that *B. coli* (Escherich) survives ten days' growth on lactose-peptone water at 37° C. and *B. coli* (Durham) 13 days. But a recently selected *B. coli* like the lactose-fermenting strain of *B. coli mutabile* (Weiss) cannot be sub-cultured after two days' growth on lactose-peptone water at 37° C. The recently selected lactose-fermenting strain is evidently much less resistant to the acid produced than the natural varieties of *B. coli* which ferment lactose without previous selection.

*Charts IV and V* show also sudden falls in the total count.

*Charts VIII and IX.* To show the march of events in the type which does not attain full acidity in the dulcite broth. Observe that the non-fermenting elements predominate throughout, though on two occasions with a rising total count, reds appeared on the plates in the case of (a), but only during the second maximum in case of (b).

## ON THE PARATYPHOID GROUP OF BACILLI.

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AND R. A. O'BRIEN, M.D., B.S., D.P.H.

(From The Lister Institute of Preventive Medicine.)

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## *Introduction.*

THERE exists at the present time considerable difference of opinion both as to the distribution in nature of *B. paratyphosus* (B) and as to its relation to disease in man. The name was given by Schottmüller (1900 and 1901) to a bacillus, which he isolated from patients whose clinical symptoms were those of enteric fever, and which he regarded as the causal organism of their illness. Further enquiry soon showed that this bacillus was closely related to *B. suis* (*pestifer* or *aertryck*) which occurs in the intestine of healthy pigs and has been found on several

occasions in association with outbreaks of food-poisoning in man. Almost all writers in this country, however, regard them as distinct organisms which can be differentiated from one another sometimes by their agglutination reactions and always, as both Boycott (1906) and Bainbridge (1909) have shown, by the absorption method; and most workers appear to have experienced no difficulty in identifying the strains which they isolated as *B. suipestifer* or *B. paratyphosus* (B) respectively. The majority of English writers therefore limit the term *B. paratyphosus* (B) to those bacilli which correspond in all respects, including their reaction to absorption tests, to original strains of the bacillus isolated by Schottmüller.

On the contrary Kutscher and Meinecke (1906), Bock (1906) and others regard *B. suipestifer* and *B. paratyphosus* (B) as indistinguishable from one another, and as being in all probability identical organisms; with the exception of a few observations by Bock, who was able to differentiate *B. paratyphosus* (B) from *B. suipestifer*, the absorption method was apparently not employed by these observers. This conclusion has been generally accepted by German writers, who almost without exception describe as *B. paratyphosus* (B) all bacilli whatever their source may be, which correspond in their cultural and agglutination reactions to the "paratyphoid" group; and they draw no distinction between *B. suipestifer* and *B. paratyphosus* (B), although they retain the term *B. suipestifer* for the organisms of this group which are derived from pigs.

#### *Distribution in man.*

Since some writers draw a distinction between *B. suipestifer* and *B. paratyphosus* (B), whereas others do not, and since the term *B. paratyphosus* (B) is not used in the same sense by different writers, it is hardly to be expected that they should agree as to the distribution and pathogenic significance of "paratyphoid" bacilli. The evidence regarding the distribution of these bacilli in man is, indeed, remarkably conflicting. On the one hand a very complete investigation of the faeces of 303 cases of summer diarrhoea by Morgan (1905-1907) yielded only two strains which gave the cultural and agglutination reactions characteristic of *B. suipestifer*. Savage (1909) examined sixty samples of faeces and urine from typhoid patients and from healthy individuals, but failed to find paratyphoid (B) bacilli in man except in cases of paratyphoid fever; Williams, Rundle and Murray (1910) examined

*B. paratyphosus Group*

the stools of 100 healthy children with completely negative results. Nor was *B. paratyphosus* (B) found either by Seiffert (1909) who examined the faeces of 600 healthy men, or by Sobernheim (1910) who investigated 1,000 samples of urine and faeces from healthy men. The conclusion, to which these observations point, is that the paratyphoid (B) bacillus is very rarely found in the human intestine or in urine apart from cases of paratyphoid fever.

On the other hand, many German workers have brought forward evidence that "paratyphoid" bacilli (in the wider sense of the term) can be obtained from the faeces, the urine and even the blood of healthy individuals. Thus, Conradi (1909) isolated "paratyphoid" bacilli from the faeces of twenty-nine out of 250 typhoid convalescents, from several typhoid patients and "contact" cases, from patients suffering from other diseases, and from healthy individuals. In the course of some years, Prigge and Sachs-Müke (1909) examined the faeces or urine (or both) of 5,252 people, and found "paratyphoid" bacilli in seventy cases, apart from cases of paratyphoid fever. Similar observations, though of a less striking character, have been published by Gaethgens (1907) and others.

*Distribution in animals and food.*

The distribution of the "paratyphoid" group of bacilli in food and in the lower animals has received hardly any attention in this country, and no systematic search for these organisms appears to have been carried out<sup>1</sup>.

Recent observations by Petrie and O'Brien (1910), however, point to the frequent occurrence of *B. suis* in healthy guinea-pigs, and O'Brien (1910) has met with several guinea-pigs which were persistent carriers of this bacillus. In the course of the bacteriological examination of 850 glucose-fermenting bacilli obtained from many samples of milk (number not stated), Orr (1908) found eight strains which gave the cultural reactions characteristic of the "paratyphoid" group, but were not subjected to agglutination tests. Observations on the presence of these organisms in meat seem to be entirely lacking.

In Germany, bacilli of the "paratyphoid" group have been found in milk by Fischer (1903), in water by Conradi (1904), Priefer and

<sup>1</sup> One of us (F. A. B.) has recently examined the intestinal contents of 50 healthy pigs; no bacilli of this group were found.

Keyser (1902), in sausages by Hübener (1908) and by Rommeler (1909), and in goose (*Spickgänse*) by Sobernheim (1910). They have also been obtained from the intestinal contents of healthy pigs by Uhlenhuth (1908) (8%) and by Seiffert (1909) (3.5%).

On the ground of this evidence and of their observation that the consumption by man of food containing these bacilli led to no ill effects, Hübener and Conradi consider not only that "paratyphoid" bacilli have a wide distribution in nature, but also that they are usually harmless for man, and that the isolation of these bacilli from the excreta of a human being is no indication that he has or has had a paratyphoid infection, and is in fact of no special significance.

It seemed to us possible that an enquiry into the distribution of *B. suispestifer* on the one hand and of *B. paratyphosus* (B) on the other hand might furnish some evidence as to their pathogenic significance, and might also assist in explaining the opposing views now held by various workers. The present investigation was carried out from this point of view.

#### *The scope of the present enquiry.*

Although the cultural characters of *B. enteritidis* (Gaertner) are identical with those of *B. suispestifer*, both *B. Gaertner* and *B. paratyphosus* (A) are clearly marked off from *B. suispestifer* and *B. paratyphosus* (B) by their agglutination reactions. They have, therefore, been left out of consideration in this paper, and the term "paratyphoid group of bacilli," as here used, includes only *B. suispestifer* and *B. paratyphosus* (B).

The investigation of the "paratyphoid" group has been carried out along two lines. In the first place, the faeces and urine of a large number of typhoid convalescents were examined in order to obtain some evidence as to the prevalence of bacilli of the "paratyphoid" group in the human alimentary canal, apart from cases of paratyphoid fever.

Secondly, a considerable number of strains of the "paratyphoid" group of bacilli was obtained from various sources. They were compared with standard, recognised strains of *B. suispestifer* and *B. paratyphosus* (B) as regards their cultural characters and their agglutination and absorption reactions. Attention was particularly directed to their absorption reactions, since as previously mentioned it has been shown by Boycott (1906) and also by Bainbridge (1909) that *B. suispestifer* and *B. paratyphosus* (B) can be readily differentiated from one another by

## B. paratyphosus Group

means of the absorption method of Castellani (1902), and that the use of this method is essential for the complete identification of any given strain belonging to the "paratyphoid" group.

### Description of the organisms examined.

The following strains have been examined:—

#### A. Standard strains.

	Description	Source
(a)	<i>B. paratyphosus</i> (B)	Schottmüller; original strain 1901.
(b)	„	Professor MeWeeney: isolated from a fatal case of paratyphoid fever.
(c)	<i>B. suis</i> f. <i>stifer</i>	Laboratory strain (Kral).
(d)	„	Isolated from a healthy pig: sent by Professor Uhlenhuth.
(e)	„	Sent by Professor Wassermann.

These strains had been repeatedly tested by the absorption method and always yielded constant results.

#### B. Fresh strains.

	Description	Source
(1)	Eisenmeyer	Paratyphoid fever case.
(2)	Bieber	Paratyphoid carrier (Dauerträger).
(3)	Kunz	„ „ „
(4)	Vogt	„ „ „
(5)	Sehnorr	„ „ „
(6)	Kant	Healthy man.
(7)	Ma	„ Sick man.
(8)	Düsseldorf	Food.
(9)	Heydr. Ali	Liver cheese (Leberkäse).
(10)	Ba	Blood sausage (Blutwurst).
(11)	Liver	Food-poisoning (patient).
(12)	Sausage	Paratyphoid fever case.
(13)	Murrow	Food-poisoning (patient).
(14)	Savage, c	Food-poisoning (patient).
(15)	„ w	Paratyphoid fever case.
(16)	Lyons	Gastro-enteritis (patient).
(17)	Winfeld	Healthy man.
(18)	Gill	Paratyphoid fever case.
(19)	Zender	Paratyphoid fever case.
(20)	Lieblang	Water.
(21)	Lister	Summer diarrhoea.
(22)	Bacillus F	Fatal Ac. gastro-enteritis (? food).
(23)	Victoria	„ „ „
(24)	Garton	

*Methods employed.*

The mode of carrying out the cultural, agglutination and absorption tests has been described in detail in a former paper (Bainbridge, 1909) and reference need be made only to two points. In the first place, some difficulty was experienced in obtaining highly agglutinating sera by the injection of *killed* cultures into rabbits, and the sera agglutinated equally both *B. suis* and *B. paratyphosus* (B). Sera, obtained from rabbits injected with *living* cultures, had a very high agglutination titre for the homologous organism, and a distinctly lower titre for heterologous strains.

Secondly, attention may be called to Dreyer's observation that the absorption of agglutinins consists partly in a specific combination of agglutinin and bacteria, and partly in a non-specific or less specific physical absorption of agglutinin. It is possible by the addition of very large amounts of a heterologous bacillus to remove some of the homologous agglutinin from a serum, but we have not succeeded in removing all the homologous agglutinin in this way. The difference between the amount of heterologous bacilli which must be added to a serum to absorb only the heterologous agglutinin, leaving the homologous agglutinin intact, and that necessary to absorb much of the homologous agglutinin, is so large that errors cannot occur if reasonable care is taken (as may be seen in Tables I and II). As a rule, one or two trials suffice to determine the amount of heterologous bacilli, which must be added to a serum in order to remove the heterologous agglutinins—and so get the specific effect—without appreciably affecting the homologous agglutinin; when this amount has been once ascertained, the result can be reproduced without difficulty on subsequent occasions. It is also safer and preferable to measure the agglutination limit of the serum after absorption rather than merely to observe the agglutination at one or two dilutions, and it is important in every experiment to test the absorbed serum against standard strains as well as against the organism under investigation. The absorption method is also much more easily carried out, and the results obtained are much sharper, when sera are used which have a high agglutination titre (10,000 or more).

The examination of the faeces and urine of the typhoid convalescents was carried out in the following manner: the material was plated out on lactose-neutral-red-bile-salt agar and a number of white colonies

## B. paratyphosus Group

were picked off into mannite broth by Dr Ledingham, who kindly handed on to us all cultures which formed acid and gas in this medium. The cultures were replated on lactose-saccharose-bile-salt-neutral-red agar, and the colourless colonies were picked off into dulcite broth. The cultures, which fermented dulcite, were then tested by all the ordinary cultural tests used for identification of bacilli of the "paratyphoid" group.

TABLE I. *The effect of progressive absorption of a serum with heterologous bacilli.*

A Paratyphoid (B) serum was absorbed with *B. suispestifer*, the bacilli being added from the agar slopes.

Paratyphoid (B) serum	Agglutination limits after absorption		
	<i>B. paratyphosus</i> (B)	<i>B. suispestifer</i>	<i>B. aertryck</i>
Original titre	... ...	4,000	1,000
1st absorption with 3 slopes	...	4,000	< 200
2nd .. .. 3 further slopes		2,000	< 100
3rd .. .. 3 .. ,		1,000	—

TABLE II. *The effect of absorbing a serum with varying amounts of heterologous bacilli.*

The volume of serum used was constant, namely 2 c.c. of 1-10 dilution; the amount of bacilli added varied from 2-8 agar slopes of approximately uniform size and density of growth.

A *B. suispestifer* serum was absorbed with *B. paratyphosus* (B).

Serum	Agglutination limits after absorption	
	<i>B. suispestifer</i>	<i>B. paratyphosus</i> (B)
<i>B. suispestifer</i> serum. Original titre	20,000	5,000
.. absorbed with 2 slopes	20,000	< 100
.. .. 4 ..	20,000	< 100
.. .. 8 ..	20,000	—

### Results.

#### (a) Examination of typhoid convalescents.

It is now generally admitted that *B. suispestifer* and *B. paratyphosus* (B) are indistinguishable by their cultural reactions. Briefly these reactions are as follows :

- (1) The production of acid and gas in broth containing glucose, mannite, dulcite, lactulose, galactose, arabinose, maltose and sorbit.

(2) No apparent change in broth containing lactose, saccharose, salicin, raffinose and inulin.

(3) Litmus milk shows an initial transient acidity not often lasting more than 24 hours; in 3—5 days the milk is definitely alkaline and at the end of a week intensely alkaline; no clot is formed, and there is no obvious sign of digestion.

(4) No indol is formed.

(5) Neutral red broth is usually reduced in 48 hours, but this test is less reliable than the others.

When subjected to these tests, not one of the 341 colonies which we obtained from the faeces or urine of approximately 300 typhoid convalescents, corresponded in its cultural characters to *B. paratyphosus* (B). A large proportion of them fermented saccharose, and the remainder produced indol and fermented salicin. Several of the strains, which most nearly resembled *B. paratyphosus* (B) in their cultural characters, were tested with Gaertner and paratyphoid (B) sera, but none of them was agglutinated in dilutions higher than 1—100 (Table III). Similar results were obtained by Savage (1909). We have in fact never met with any organism which was agglutinated beyond the above limit by a paratyphoid (B) (or Gaertner) serum, unless its cultural and fermentation characters corresponded in all respects with those bacilli.

TABLE III. Agglutination reactions of strains isolated from typhoid convalescents. These strains resembled but were not completely identical with *B. paratyphosus* (B) in their cultural reactions.

Strain	Paratyphoid serum (titre 20,000)	Gaertner serum (titre 5,000)
1. Lythe	< 100	< 100
2. Crowdÿ	< 100	< 100
3. Newman	< 100	< 100
4. B. B. 3	< 100	—
5. K. 4	< 100	—
6. Roberts, 2	< 100	—
7. Robinson	< 100	—
8. B. 270	< 100	< 100

These results entirely confirm those of Morgan (1907), Seiffert (1909), Savage (1909), Williams, Rundle and Murray (1910), as opposed to those of Conradi (1909), Gaethgens (1907) and Prigge and Sachs-Müke (1909). There is no reason to suppose that the differences in the technique adopted by different writers for the isolation of this group of bacilli account for these conflicting results. The differences consist

almost entirely in the medium used for plating out the material under investigation; some workers used lactose-bile-salt-neutral-red agar, others use malachite green and Conradi-Drigalski agar.

A partial explanation may perhaps be found in the fact that some workers appear not to make use of dulcite, saccharose or salicin for the identification of "paratyphoid" bacilli, and may in some cases describe as *B. paratyphosus* (B) strains which would be excluded by others, who apply more extensive cultural tests. One strain sent to us as *B. paratyphosus* (B) differed widely in its cultural characters from standard strains of *B. paratyphosus* (B), and did not agglutinate (beyond a dilution of 1 in 40) with paratyphoid and *suipestifer* sera. Morgan (1907) has shown clearly that organisms occur with considerable frequency in the human intestine, which in some respects resemble "paratyphoid" bacilli, but which can be distinguished from them by adequate cultural and fermentation tests.

It is not improbable, however, that actual differences in the regional distribution of these organisms largely account for the apparently discordant observations of various writers. Although bacilli of the "paratyphoid" group have very rarely been isolated (apart from cases of paratyphoid fever) in London, Williams, Rundle and Murray (1910), working in Liverpool, obtained *B. suipestifer* from seven (probably seventeen) cases of summer diarrhoea. Again Sobernheim (1910) only occasionally found paratyphoid bacilli in Berlin, either in food or in healthy men, whereas Conradi (1909) frequently isolated these organisms from typhoid convalescents and healthy men in Western Germany.

Whether the distribution of these bacilli is such that in some districts they are frequently obtained both from food and from man, and in other districts they are rarely met with in food or in man, has yet to be proved. But the available evidence certainly suggests that such may be the case.

(b) *Examination of strains of the paratyphoid group.*

(1) *Cultural characters.*

All the strains gave the cultural reactions characteristic of the "paratyphoid" group of bacilli, except No. 20 (Lieblang), which fermented no sugars and was probably *B. faecalis alkaligenes*.

(2) *Agglutination reactions.*

Four sera were systematically employed for the carrying out of the agglutination tests, and in addition other sera were occasionally used.

The results, which are given in Table IV, show that with one or two exceptions all the strains were agglutinated equally well by the sera (A and B) obtained from rabbits, which had been injected with killed cultures of *B. suis* and *B. paratyphosus* (B). In the case of the sera (C and D) obtained by the injection of living cultures into rabbits the agglutination limits were not the same for all strains and some indication was revealed of the existence of two types of bacilli. Some strains behaved like the standard strains of *B. suis*, others like

TABLE IV. Agglutination reactions of the strains.

Organisms	Source	Serum			
		From rabbits injected with killed cultures		From rabbits injected with living cultures	
		A <i>B. suis</i> Wassermann	B <i>B. paratyphosus</i> (B) Schottmüller	C <i>B. suis</i> McWeeney	D <i>B. paratyphosus</i> (B) McWeeney
<b>A. Standard strains.</b>					
(a) <i>B. paratyphosus</i> (B)	Schottmüller (1901) original strain	4,000	2,000	5,000	20,000
(b) " "	From Prof. McWeeney ...	4,000	2,000	5,000	20,000
(c) <i>B. suis</i>	Laboratory strain ...	4,000	2,000	10,000	10,000
(d) "	From Prof. Uhlenhuth ...	4,000	2,000	10,000	5,000
(e) "	From Prof. Wassermann ...	--	--	10,000	10,000
<b>B. Strains investigated.</b>					
(1) Eisenmeyer	Paratyphoid fever case ...	2,000	2,000	5,000	20,000
(2) Bieber	" " carrier (Dauerträger)	4,000	2,000	10,000	20,000
(3) Kanz	" " "	2,000	1,000	5,000	20,000
(4) Vogt	" " "	4,000	2,000	5,000	20,000
(5) Schnorr	" " "	4,000	2,000	5,000	20,000
(6) Kant	Healthy man ...	4,000	2,000	5,000	20,000
(7) Ma	" " ...	4,000	2,000	5,000	20,000
(8) Düsseldorf	Food-poisoning (patient)	4,000	100	10,000	< 400
(9) Heydr. Ali	" Sick man " ...	2,000	2,000	10,000	5,000
(10) Ba	Food ...	4,000	2,000	10,000	5,000
(11) Liver	Liver cheese (Leberkäse)	4,000	1,000	10,000	5,000
(12) Sausage	Blood sausage (Blutwurst)	4,000	2,000	10,000	5,000
(13) Murrow	Food-poisoning (patient)	4,000	100	10,000	< 200
(14) Savage, c	Paratyphoid fever case ...	4,000	2,000	5,000	20,000
(15) " w	" " ...	--	--	5,000	20,000
(16) Lyons	Acute gastro-enteritis ...	2,000	1,000	< 1,000	< 200
(17) Winfield	Paratyphoid fever case ...	2,000	2,000	5,000	10,000
(18) Gill	Acute gastro-enteritis ...	4,000	2,000	10,000	20,000
(19) Zender	Healthy man ...	1,000	1,000	10,000	2,000
(20) Lieblang	Paratyphoid fever case ...	100	< 100	< 100	< 200
(21) Lister	Water supply ...	--	--	2,000	20,000
(22) Bacillus F	Acute gastro-enteritis ...	--	--	10,000	5,000
(23) Victoria	" " ...	--	--	10,000	5,000
(24) Garton	" " ...	--	--	10,000	5,000

## B. paratyphosus Group

standard strains of *B. paratyphosus* (B). It was also noticed in the case of serum D, that some strains were agglutinated into large clumps leaving a clear surrounding fluid, whereas other strains, even in such low dilution of the serum as 1 in 100, formed minute clumps, which had little or no tendency to settle and leave a clear fluid. The absorption method made it clear that with serum D and to a less degree with other sera, homologous bacilli tended to form large clumps, while heterologous bacilli formed small clumps. This difference has also been observed by Seligmann and Sobernheim (1910), although they did not attribute any significance to the observation.

As might be expected from its cultural characters, strain 20 (Lieblang) was not agglutinated by any of the sera in dilutions higher than 1 in 100. It has in fact been our experience that no bacillus which is not in all respects identical in cultural characters with *B. suispestifer* (or *B. paratyphosus* (B)) is ever agglutinated beyond dilutions of 1 in 100 by a *suispestifer* or *paratyphosus* (B) serum. We have however met with two organisms, i.e. Nos. 16 and 19, which although identical in cultural characters with "paratyphoid" bacilli agglutinated rather feebly with our sera as compared with standard strains of *B. suispestifer* and *B. paratyphosus* (B).

It may be added that none of our sera agglutinated *B. typhosus*, *B. enteritidis* Gaertner, or *B. paratyphosus* (B) in higher dilutions than 1 in 100.

### (3) Absorption tests.

The absorption method was introduced by Castellani (1902) and was at first used mainly for the diagnosis of mixed infections. An obvious extension of the principle underlying the absorption method is that, if all the agglutinins formed in response to vaccination with a given organism are removed from the serum not only by it but also by some heterologous organism, the two organisms must be identical.

The method, if employed for a number of strains, is very laborious and in the case of the "paratyphoid" group of bacilli it can be used in a much simpler form. It has been shown that the members of this group of bacilli fall into two and only two classes, namely, one corresponding in all respects to *B. suispestifer* (or *aertryck*) and one corresponding to *B. paratyphosus* (B). It follows, therefore, that a serum which has been absorbed by the heterologous organism, is all that is needed for the identification of an unknown strain belonging to this group. Thus if a *paratyphosus* (B) serum is absorbed with *B. suispestifer* until the heterologous agglutinin is removed, it will still agglutinate

*paratyphosus* (B) but not *B. suis*, and by the use of such a serum it is perfectly simple to identify an unknown strain, since an organism which gives the correct cultural reactions and is agglutinated in high dilution by *paratyphosus* (B) or *suis* sera, can only be *B. suis* or *B. paratyphosus* (B), so far as is known. It was found unnecessary to absorb the sera with all the unknown organisms, and we have used almost entirely *B. paratyphosus* (B) sera absorbed with *B. suis*, and *B. suis* sera absorbed with *B. paratyphosus* (B). We have however checked our results for some strains by absorbing different sera with unknown strains according to the original method of Castellani.

The method of partial absorption was found to be very satisfactory and the results were concordant; no discrepancy was revealed when the sera were absorbed by unknown strains. It is desirable nevertheless to remove the heterologous agglutinin very completely since different strains vary slightly in the readiness with which they absorb agglutinin from, and are agglutinated by, a given serum. This variation represents nothing more than a certain individuality among members of the same group of bacilli, and is in no way comparable to the distinction between *B. suis* and *B. paratyphosus* (B). The bacillus "F," recently described by Williams, Rundle and Murray (1910), is an interesting example of such individuality. These authors obtained from cases of summer diarrhoea seven strains, three of which were identical with *B. suis*, whereas four gave anomalous absorption reactions, and were regarded by the writers as being intermediate between *B. suis* and *B. paratyphosus* (B) and were called bacillus "F." Dr Williams very kindly furnished us with a strain of this organism, and also a serum homologous for it. We found that the bacillus gave the cultural tests characteristic of the "paratyphoid" group. Agglutination and absorption tests gave the following results, the organism being compared with recognised strains of *B. paratyphosus* (B) and *B. suis*:

TABLE V. *Agglutination reaction. Macroscopic Method.*

Serum	Bacillus F	Organisms	
		Lab. strain <i>B. suis</i>	<i>Paratyph.</i> (B) (McWeeney)
1. <i>B. suis</i>	...	10,000	10,000
2. <i>B. paratyphosus</i> (B) (McWeeney)	...	5,000	10,000
3. Bacillus F	...	5,000	5,000
4. <i>B. enteritidis</i> Gaertner (titre 5,000)	<100	<100	<100

## B. paratyphosus Group

TABLE VI. *Absorption tests.*

	Serum	Agglutination limits after absorption		
		Bacillus F	<i>B. suispestifer</i>	Paratyph. (B) (McWeeney)
<b>1. <i>Bacillus F.</i></b>				
Original titre...	...	5,000	5,000	2,000
Abs. with Bacillus F	...	<200	<200	<200
" <i>B. suispestifer</i> ...		<200	<200	<200
" <i>B. paratyph.</i> (B)		5,000	5,000	<200
<b>2. <i>B. suispestifer.</i></b>				
Original titre...	...	10,000	10,000	5,000
Abs. with <i>B. paratyph.</i> (B)		10,000	10,000	<200
" Bacillus F ...		<50	<50	<50
<b>3. <i>B. paratyphosus</i> (B).</b>				
Original titre...	...	5,000	10,000	20,000 ;
Abs. with <i>B. suispestifer</i> ...		<400	<400	10,000
" Bacillus F ...		<400	<400	10,000 ,

It is perfectly clear that Bacillus F is indistinguishable from recognised strains of *B. suispestifer* and that the slight differences noted by Williams, Rundle and Murray disappear when the sera are fairly completely absorbed and the agglutination limits are subsequently determined. These authors<sup>1</sup> have accepted the results of our observations upon Bacillus F and also our interpretation of the slightly different results which they obtained; and they now regard Bacillus F as identical with *B. suispestifer* and not as a separate strain.

The general results obtained by the absorption method are set forth in Tables VII—X<sup>2</sup>. Table VII shows the results obtained with recognised standard strains of the *B. suispestifer* and *B. paratyphosus* (B). The strains obtained for investigation have been arranged in three groups, corresponding to their mode of origin, so far as it is known.

Group I (Table VIII) includes all strains obtained from food or from outbreaks of illness, clinically resembling acute food-poisoning. Group II (Table IX) includes all the strains obtained from cases of paratyphoid fever, and from persistent paratyphoid carriers (Dauerträger). Group III (Table X) contains strains concerning which information is inadequate or absent.

<sup>1</sup> Private communication kindly made by Dr Williams.

<sup>2</sup> Absorption tests were also made with the sera A and B; the results have been omitted since they were identical with those obtained with sera C and D, which are recorded in Tables VII—X.

With two exceptions, the strains included in Group I are identical with the standard strains of *B. suispestifer*. Strain No. 16 was obtained from a severe outbreak of food-poisoning caused apparently by infected goose-flesh. It has all the cultural characters of the "paratyphoid" group, but agglutinates very slightly with *suispestifer* and *paratyphosus* (B) sera. As far as they go, its agglutination reactions suggest that it is a strain of *B. suispestifer*, but it agglutinates so feebly that absorption tests were of no help in identifying it. This is the only strain we have found to be culturally identical with *B. suispestifer*, but which was agglutinated only slightly by *suispestifer* and *paratyphosus* (B) sera.

Strain No. 18 is extremely interesting. It was obtained from the faeces of patients suffering from acute gastro-enteritis, clinically resembling food-poisoning. Subsequent enquiry showed that the outbreak

TABLE VII. *Absorption test.*

## Examination of standard strains.

Strains examined	<i>B. suispestifer</i> serum.		<i>B. paratyphosus</i> (B) serum.			
	Agglutination titre before and after absorption with <i>B. paratyphosus</i> (B)	Original titre	Titre after absorption	Agglutination titre before and after absorption with <i>B. suispestifer</i>	Original titre	Titre after absorption
(a) <i>B. paratyph.</i> (B); Schottmüller	5,000	< 200	20,000	10,000		
(b) " McWeeney ...	5,000	< 100	20,000	10,000		
(c) <i>B. suispestifer</i> ; laboratory strain	10,000	5,000	10,000	< 200		
(d) " Uhlenhuth ...	10,000	5,000	5,000	< 200		
(e) " Wassermann ...	10,000	5,000	10,000	< 400		

TABLE VIII. *Absorption test.*

## GROUP I. Examination of strains obtained from food or from cases of acute gastro-enteritis.

Strains examined	<i>B. suispestifer</i> serum.		<i>B. paratyphosus</i> (B) serum.			
	Agglutination titre before and after absorption with <i>B. paratyphosus</i> (B)	Original titre	Titre after absorption	Agglutination titre before and after absorption with <i>B. suispestifer</i>	Original titre	Titre after absorption
(1) No. 10. Food ...	... 10,000	5,000	5,000	< 400		
(2) No. 11. " ...	10,000	5,000	5,000	< 400		
(3) No. 12. " ...	10,000	5,000	5,000	< 400		
(4) No. 13. Food poisoning ...	10,000	5,000	< 200	< 200		
(5) No. 8. " "	10,000	5,000	< 400	< 400		
(6) No. 18. Ac. gastro-enteritis	10,000	< 200	20,000	10,000		
(7) No. 22. " "	10,000	5,000	5,000	< 400		
(8) No. 23. " "	10,000	10,000	5,000	< 200		
(9) No. 24. " "	10,000	10,000	5,000	< 200		
(10) No. 16. " "	< 1,000	< 400	< 200	—		

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TABLE IX. Absorption test.

GROUP II. Strains from cases of paratyphoid fever or persistent "paratyphoid" carriers.

Strains examined	<i>B. suispestifer</i> serum.		<i>B. paratyphosus</i> (B) serum.			
	Agglutination limits before and after absorption with <i>B. paratyphosus</i> (B)	Original titre	Titre after absorption	Agglutination limits before and after absorption with <i>B. suispestifer</i>	Original titre	Titre after absorption
(1) No. 1. Paratyphoid fever...	5,000	<200	20,000	10,000		
(2) No. 2. , carrier	10,000	100	20,000	10,000		
(3) No. 3. , "	5,000	<400	20,000	10,000		
(4) No. 4. , "	5,000	<400	20,000	10,000		
(5) No. 5. , "	5,000	<200	20,000	10,000		
(6) No. 14. , fever	5,000	<400	20,000	10,000		
(7) No. 15. , "	5,000	<400	20,000	10,000		
(8) No. 17. , "	5,000	<400	10,000	10,000		
(9) No. 20. , "	<400	--	<200	--		

TABLE X. Absorption test.

GROUP III. Strains whose origin and pathogenic relationship are unknown or doubtful.

Strains examined	<i>B. suispestifer</i> serum.		<i>B. paratyphosus</i> (B) serum.			
	Agglutination limits before and after absorption with <i>B. paratyphosus</i> (B)	Original titre	Titre after absorption	Agglutination titre before and after absorption with <i>B. suispestifer</i>	Original titre	Titre after absorption
(1) No. 6. Healthy man ...	5,000	<400	20,000	10,000		
(2) No. 7. , , ...	5,000	<200	20,000	10,000		
(3) No. 19. , , ...	10,000	<100	5,000	<200		
(4) No. 9. "Sick" man ...	10,000	10,000	5,000	<200		
(5) No. 21. Water ...	2,000	<200	20,000	10,000		

had not been caused by diseased food, but was possibly brought about by a human paratyphoid carrier, and that it is not a genuine example of food-poisoning.

All the strains included in Group II are identical with *B. paratyphosus* (B), except No. 20, which was not agglutinated by *suispestifer*, *paratyphosus* (B) (or Gaertner) sera, although its cultural characters were identical with those of *B. paratyphosus* (B).

No definite history of the strains included in Group III could be obtained. Three were from healthy men, but it was not known whether they were true paratyphoid carriers, or whether they had recently suffered from paratyphoid fever or food-poisoning. One was from a "sick" man, but the nature and causation of the illness could not be ascertained by us. Strain No. 21 was isolated from a well water which

was otherwise bacteriologically satisfactory: no further information could be obtained with regard to it, except that attacks of jaundice had occurred among those who drank the water.

It thus appears that with two exceptions (Nos. 16 and 19) all the strains which we examined could be identified without difficulty by the absorption method as either *B. suispestifer* or *B. paratyphosus* (B). Every strain which was agglutinated by *suispestifer* and *paratyphoid* sera gave perfectly sharp and definite results when tested by the absorption method. The strains, which were obtained from undoubted cases of *paratyphoid* fever or persistent carriers, were all identical with standard strains of *B. paratyphosus* (B), while the organisms obtained from food or from outbreaks of illness definitely attributable to infection by food were identical with *B. suispestifer*.

#### *Discussion of results.*

There seems to be no doubt that those strains of bacilli which, by virtue of their cultural characters and agglutination reactions, belong to the *paratyphoid* group, can be further separated into two classes by means of the absorption method<sup>1</sup>. We regard the distinction which is revealed by this method as a real one, and we consider that, although they are closely related, *B. suispestifer* (with which *B. aertryck* is identical) and *B. paratyphosus* (B) are different organisms.

It has now been shown that *B. paratyphosus* (B) causes not only *paratyphoid* fever, but also sudden acute illness (Bainbridge and Dudfield, 1911); and the distinction formerly drawn on clinical grounds between infection by *B. suispestifer* and by *B. paratyphosus* (B) can no longer be maintained. Nevertheless, the distinction between these two organisms is of importance from an epidemiological point of view, since they appear to have a different distribution in nature.

Although we have not examined a very large number of strains, all the strains of *B. paratyphosus* (B) in our series were derived from man. We are disposed to regard the human alimentary canal (including the bile passages) as the normal habitat of *B. paratyphosus* (B) while admitting that *paratyphoid* "carriers" may cause infection not only directly but also indirectly by contaminating otherwise sound food,

<sup>1</sup> Dr H. R. Dean has examined a number of the strains of our series by the complement deviation method; his results confirm our observations and show that strains of *B. suispestifer* and *B. paratyphosus* (B) can be differentiated from one another. (Private communication.)

just as is the case with typhoid carriers. Under these circumstances it is to be expected that the bacilli may occasionally be found in food, polluted by a carrier, although we have not met with such an instance.

Lentz (1905) appears to have been the first to recognise the existence of persistent paratyphoid carriers; in 1905, he recorded five cases, one of whom had possibly given rise to a small epidemic of paratyphoid fever. During the investigation of a typhoid epidemic, Wernicke (1907) found a paratyphoid carrier, who was employed in a milk shop; and the evidence suggested that a number of cases of paratyphoid fever were caused by this carrier.

More recently, Prigge (1909) has described several persistent carriers, whose serum agglutinated paratyphoid bacilli, and who had either suffered from paratyphoid fever, or had been in contact with cases of paratyphoid fever. Strains Nos. 2, 3, 4, and 5, of our series were obtained from these cases. The frequency in carriers of disease of the bile-passages is also of interest. Hamilton (1910) examined twenty-four cases of chronic cholecystitis and found five carriers, and Gaethgens (1907) met with two carriers, who were suffering from chronic cholelithiasis; almost all these carriers were women. No paratyphoid carrier has yet been recorded in this country although the outbreak of illness, from which strain No. 18 was obtained, was thought, mainly on epidemiological grounds, to be caused by a carrier<sup>1</sup>.

The part played by carriers in the causation of paratyphoid fever has still to be worked out, but it seems possible that careful search for carriers among those in actual contact with cases of paratyphoid fever may furnish a clue to the epidemiology of that disease.

In contrast to *B. paratyphosus* (B), it appears from our observations that the usual habitats of *B. suis* are first the alimentary canal of the pig and perhaps other domestic animals, and, secondly, meat derived from such animals. No unimpeachable evidence has been brought forward as to the prevalence in this country of *B. suis* in food or in the lower animals. The observations of Morgan<sup>2</sup> (1905), who obtained this organism by injecting into guinea-pigs mixed cultures from the faeces of pigs, are open to criticism, since Petrie and O'Brien (1910) have shown that it may often be obtained from healthy guinea-

<sup>1</sup> A recent epidemic of gastro-enteritis at Wrexham is attributed to infection by a paratyphoid carrier. (See p. 89 this Journal. Ed.)

<sup>2</sup> Dr Morgan himself now regards the results of these experiments as inconclusive. (Private communication.)

pigs; and there is other evidence that it may be obtained from guinea-pigs inoculated with material in which this bacillus was certainly not present.

In Germany, however, both Uhlenhuth (1908) and Seiffert (1909) have obtained *B. suispestifer* from the alimentary canal of the pig in a fair percentage of cases; and the strains Nos. 10, 11 and 12 of our series, which were isolated from apparently healthy food by German workers, were identical with standard strains of *B. suispestifer*.

Although the recent observations of Sobernheim (1910) and of Zwick and Weichel (1910) seem to show that bacilli of the "paratyphoid" group are much less often present in food (in some parts of Germany) than was at first supposed, there is no doubt of their occurrence in apparently healthy food. Under these circumstances they must, from time to time, be ingested by man, although their stay in the human alimentary canal is apparently very transitory. Thus Conradi (1909) found that when meat containing these bacilli was eaten by man, they could be obtained from the faeces on the following day, but not subsequently. Prigge (1909) also noticed that individuals who ate such meat, showed no evidence of infection, and that the transitory occurrence of bacilli of the "paratyphoid" group in their alimentary canal did not increase the agglutinating power of their blood for these bacilli. It may be noted that persistent *B. suispestifer* carriers have not been observed in man. If, as our observations lead us to suppose, these writers were dealing with strains of *B. suispestifer* it would appear that the isolation on a single occasion of *B. suispestifer* from the faeces of a healthy man, whose serum does not agglutinate the organism in higher dilutions than that of normal individuals, has no pathological significance.

Nevertheless, outbreaks of acute gastro-enteritis have occurred (Durham, 1898, and De Nobele, 1898) in which the epidemiological evidence pointed to infection by food, in which *B. suispestifer* was isolated both from the food and from the excretions or organs of the patients, and in which the serum of the patients agglutinated this organism in high dilutions. Such evidence that *B. suispestifer* is capable of causing acute infection cannot be disregarded, although the rôle of this organism in swine fever may arouse scepticism as to its capacity to initiate an infection. In swine fever and epizootics among guinea-pigs, it appears to be merely a secondary invader and not primarily a cause of the infection<sup>1</sup>; and it would be a matter of great

<sup>1</sup> Uhlenhuth and Hübener (1908); Petrie and O'Brien (1910).

interest if *B. suispestifer* should prove to occupy a similar subsidiary position in relation to "food-poisoning" outbreaks in man.

Further investigation is needed in order to account for the occurrence of this organism in man at one time as an apparently harmless saprophyte, at another in association with acute gastro-enteritis.

#### SUMMARY AND CONCLUSIONS.

1. The term "paratyphoid group of bacilli" has been confined in the present enquiry to those strains which in their cultural characters and agglutination reactions are indistinguishable from *B. suispestifer* and *B. paratyphosus* (B). *B. enteritidis* Gaertner and *B. paratyphosus* (A) are excluded.

2. The strains of bacilli belonging to the "paratyphoid" group can be separated into two classes by means of the absorption method, namely one group identical with recognised, standard strains of *B. suispestifer* and one identical with standard strains of *B. paratyphosus* (B); and we regard these two bacilli as separate organisms.

3. In our experience, *B. suispestifer* has been found only in food or in outbreaks of acute illness attributable to food-poisoning, whereas *B. paratyphosus* (B) has been found in cases of paratyphoid fever or in persistent paratyphoid "carriers."

4. We are disposed to put forward the suggestion indicated by these observations, that these two organisms have a different distribution in nature, the normal habitat of *B. suispestifer* being the alimentary canal of the pig (and other animals) and of food derived from such animals, whereas the normal habitat of *B. paratyphosus* (B) is the human alimentary tract (including the gall-bladder).

5. The examination of cultures from the faeces and urine of 300 typhoid convalescents was completely negative, neither *B. suispestifer* nor *B. paratyphosus* (B) being obtained. These observations confirm those of other writers, and, apart from carrier cases, the occurrence of *B. paratyphosus* (B) in healthy human beings appears to be unknown in this country.

Our thanks are due to Drs Fisher, Trautmann, Uhlenhuth, Williams, Rommeler, Prigge, Schern, Friedrichs, Savage and Fowler, who have very kindly supplied us with strains of bacilli for investigation.

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## A SEVERE OUTBREAK OF FOOD INFECTION CAUSED BY A PARATYPHOID CARRIER<sup>1</sup>.

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THE outbreak of food infection which we are about to describe is of interest from more than one point of view. Not only was it possible to determine the organism which caused the outbreak and to trace its source to a paratyphoid carrier, but the bacteriological results yielded an important contribution to the difficult question of the differentiation of *Bacillus paratyphosus* B. and *B. enteritidis*, which for some years has been much discussed among bacteriologists<sup>2</sup>.

The outbreak of the epidemic in question, of which full particulars are given in *The Journal of The Royal Institute of Public Health* for December 1910, occurred in the beginning of August 1910 in Wrexham and its neighbourhood. More than a hundred persons were affected, and five of the sufferers died. The symptoms were those of very severe meat poisoning, such as sickness, vomiting, much abdominal pain, diarrhoea, great weakness, etc.

It was easily found that all these persons had eaten pork pies originating from the same baker. The pies in question were all manufactured on the 5th of August, and sold on the 6th. The symptoms for the most part appeared about 12 hours after the pies were eaten.

As suspicion was fixed on the pies as the cause of the illness, the Medical Officer of Health of the district (Dr Llewelyn Williams) took

<sup>1</sup> Those of our experiments which are purely of bacteriological interest will be published elsewhere.

<sup>2</sup> One of us has made similar investigations on the pathogenicity of *Bacillus typhi murium* to man, which are confirmed by the results of this inquiry. *Vide* R. Trommsdorff (1906), *Archiv für Hygiene*, Vol. LV. p. 279.

steps to obtain one of the suspected pies (Pie 1) for investigation, besides a portion of a pie (Pie 2) the remainder of which had caused the death of a person. These specimens were at once sent to the laboratories of The Royal Institute of Public Health.

It may be mentioned here that investigations in the Chemical Department of the Institute failed to detect any mineral or other poison in either specimen of pie, or in any of the ingredients subsequently received.

Both from Pies 1 and 2 emulsions were made. From these, direct cultures were taken, mice and rats fed, and mice, rats, and guinea-pigs injected. All of these animals died, with the exception of the rats. Their organs and blood throughout contained bacteria, which yielded colonies behaving in all respects like those in cultures obtained directly from the pies, growing blue on Drigalski, remaining colourless on Endo, and being easily recognisable as representatives of the hog-cholera group, by their motility, form, and cultural tests (no indole, gelatin not liquefied, gas and acid in glucose, no fermentation in milk and cane-sugar, fluorescence in neutral-red agar, formation of alkali in litmus whey), as well as by agglutination tests. The same bacillus could be cultivated from the blood and organs of mice and guinea-pigs which had been injected with the bacilli obtained from Pie 1, and from mice which had been fed with the same, and which had all died.

Further, from an elderly woman, who had been ill some 14 days and subsequently died, the heart, blood, organs, and faeces were submitted for examination. All the organs were found to be congested, and in the small intestine and caecum many superficial ulcers were present. The bacteriological investigation of the blood obtained from the heart yielded a pure culture of the same bacillus which had been found in Pies 1 and 2. Drigalski plates made from the various organs and faeces showed numerous blue colonies. On account of the wealth of material thus obtained the identity only of the colonies cultivated from the liver was exactly determined.

Further, the several specimens of blood from patients who had suffered were received for the agglutination test. The results of this test, which was carried out with different bacterial strains, can be seen in Table A. Four of the specimens (1—4) gave strong positive results, the fifth (No. 5) gave a strongly suspicious reaction.

The sera of persons who had eaten mutton pies from the same bakehouse, and who were said to have had abdominal pain, were regarded as a control.

TABLE A.

Specimens of serum taken 18. VIII. 10	Specimens of serum from sick persons who had eaten pork pies					Specimens of serum from persons who had eaten mutton pies			
	Serum No.:- Register No.:- 328	1 329	2 331	3 331	4 332	5 330	6 369	7 371	8 373
(1) Culture No. 1 (direct culture from Pie 1) ...	—	—	—	—	—	—	—	—	—
(2) Culture No. 5 (culture from heart blood of guinea-pig injected with direct culture from Pie 1 (culture No. 1))	2500	1280	160	320	80	—	—	—	—
(3) Culture No. 11 (from heart blood of guinea-pig injected with direct culture from Pie 2) ...	...	—	1280	160	320	80	—	—	—
(4) Culture No. 17 (from heart blood of fatal human case)	—	—	—	—	—	—	—	—	—
(5) <i>B. paratyphosus</i> B. <sup>1</sup> ...	320	1280	160	320	80	—	—	—	—
(6) <i>B. enteritidis</i> <sup>1</sup> ...	40	160	160	320	80	—	—	—	—
(7) <i>B. paratyphosus</i> A. <sup>1</sup> ...	40	40	40	160	40	—	—	—	—

A more detailed discussion of these results will follow.

We further received specimens of blood from three relatives of the person who died, and whose blood and organs had been sent for examination. These persons had assisted in the nursing of the deceased woman and, not having eaten any of the pies, were attacked with symptoms exactly similar to those of the woman they had nursed. Further, the son of the deceased woman died after some 14 days' illness, the death certificate giving gastro-enteritis as the cause. It was not possible for us to obtain in this latter case any postmortem material or excrement.

The results of the agglutination tests in the three attendants are given in Table B.

TABLE B.

Specimens of blood taken 13. IX. 10	Specimens of serum of persons who had nursed a fatal case, and had been attacked with a similar illness			
	Serum No.:- Register No.:- 393	1 393	2 394	3 395
(1) Culture No. 1 (direct from Pie 1) ...	...	60	240	60 + 120 ±
(2) Culture No. 5 (from heart blood of guinea-pig injected with direct culture from Pie 1 (culture No. 1)) ...	...	60	240	120 + 240 ±
(3) Culture No. 17 (from heart blood of fatal human case) ...	...	60	60	120 + 240 ±
(4) <i>B. paratyphosus</i> B. <sup>1</sup> ...	...	60	120	120 + 240 ±

Two of the sera gave a strongly positive result.

<sup>1</sup> We are indebted for these strains to the courtesy of Dr Prausnitz of the Metropolitan Asylums Board.

From this it is clear that the organisms found in the two specimens of suspected pork pies, and which were identical with that obtained from the heart blood and liver of the deceased person, were members of the hog-cholera group.

Further, the specific agglutinations for these bacteria, as well as for typical representatives of the hog-cholera group, were detected in the blood of five persons who had partaken of the pies, and who suffered in consequence, as well as in two other persons who had not eaten any of the pies but who had been engaged in nursing a fatal case consequent upon such consumption.

It is therefore established that the organisms isolated from the pork pies were the cause of the outbreak.

It is interesting to note the evidence afforded of contact infection by the illnesses affecting the three attendants on the deceased person, and the strong probability of the fatal case of the son being likewise attributable to the same cause.

No cases of illness of a typhoid character have, so far as is known, occurred since August in Wrexham or its vicinity.

The important question now arises, How did these bacilli reach the pies? There are, naturally, many possibilities. The first was to suspect the meat, and attribute the origin of the infection to a septicaemic animal. Samples of the meat used were not obtainable, but the investigations of the Medical Officer of Health strongly tended to exclude this hypothesis. The meat came from a butcher who had killed 15 pigs at the same time, pieces of meat from all being mixed and put through a mincing machine together. The product was delivered to different customers, and among them to this particular baker who had issued the infected pies. No symptoms of illness were caused by pies manufactured by any of the other customers of the butcher. It would be remarkable—if there had been a septicaemic animal—that all the infected meat should have been sent to the one baker. But of course such a possibility would not have been disregarded had it not been that another source of infection was detected.

Other ingredients used in the manufacture of the pies were lard, gelatin, and flour. We received samples of each, and these were tested culturally and by animal experiments (4 mice were fed with each specimen, and 4 guinea-pigs and 4 mice were injected with each). The lard was found to be sterile. In the flour the *Bacillus coli communis* could be detected. In the gelatin a bacillus pathogenic and toxic to mice and rabbits was detected; this however is of purely

bacteriological interest. (The bacillus liquefied gelatin and behaved otherwise culturally as a *B. paratyphosus* B.)

We were unable to suspect any one of the raw ingredients from which the pie had been made, but even if an organism belonging to the hog-cholera group had been found, no conclusion could have been drawn as we know, from the work of Uhlenhuth and his collaborators, that such bacteria are occasionally ubiquitous.

Another source of the infection of the pies was therefore sought, and this in the direction that during or after their preparation the most probable infection was by someone in the bakehouse. Specimens of the blood of all persons engaged in the bakehouse were therefore obtained, with the view of determining their agglutination results (Table C).

TABLE C.

Specimens of blood taken 27. VIII. 10	Specimens of serum of the persons engaged in the bakehouse				
	Serum No.:-	1	2	3	4
	Register No.:-	337	338	339	345
(1) Culture No. 1 (direct from Pie 1) ...	...	—	—	20	120
(2) Culture No. 5 (from heart blood of guinea-pig injected with culture No. 1) ...	...	—	160	20	—
(3) Culture No. 17 (from heart blood of fatal human case) ...	...	—	80	20	—
(4) <i>B. paratyphosus</i> B. "Prausnitz"	...	—	160	20	—
					60

All gave negative reactions except in two cases, the serum of a boy (No. 2, 338) and of the head cook (No. 4, 345).

These two persons were consequently suspected of being possible carriers, and of having caused the infection. It was desirable, therefore, that their faeces and urine should be examined. This was first done in the case of the boy who had recently commenced work at the bakery, but with negative results after repeated examinations. The boy had, however, eaten the pies in question, and the agglutination results can be attributed to the bacilli obtained from the pie thus eaten growing in the boy's organs, etc.

The examination of the excreta of the head cook gave abundant evidence of the presence of bacilli, which proved to belong to the hog-cholera group, and which were apparently identical with those in the pies and in the heart blood of the fatal human case. *Thus the blood of the head cook—who was said not to have eaten any of the pies, and who had not been ill in the least degree—gave a strong positive reaction, and eight weeks after the epidemic she was found to be excreting members of the*

*hog-cholera group in her faeces. She might, therefore, be considered as a chronic bacillus carrier, and the direct cause of the infection.*

The head cook had apparently never passed through any typhoid-like illness, nor had she so far as is known ever caused a like outbreak before. As she left the bakery at once, it was not possible to obtain a second specimen of her stools. From the urine of this woman (who had long suffered from cystitis) we likewise obtained a large number of apparently typhoid-like bacteria in pure culture.

We also obtained these same organisms from the specimen of Pie 1. It seems probable that the woman had transmitted her entire specific flora to the pies, although she was supposed personally to have had nothing to do with their making. How this transmission actually occurred it is of course impossible to say.

With regard to matters concerning the exact nature of the bacteria, which bore a causal relation to the cause of the outbreak, we propose to deal with them in more detail after further experimental work, but it must here be remembered that the *hog-cholera* group contains organisms apart from those which are essentially pathogenic to animals (*B. typhi murium*, *B. snipestifer*, *B. psittacosis*, etc.), the *B. paratyphosus* B., *B. enteritidis* Gaertner, and others.

Without doubt the Group *Enteritidis* can be differentiated, by the agglutination test, from the Group *Paratyphosus* B., *B. snipestifer*, *B. psittacosis*, *B. typhi murium*, etc. But this is not possible in all cases, or at least not without difficulty, as the agglutination test is somewhat uncertain for the exact differentiation of bacteria of these two groups, this being a difficulty which cannot easily be overcome. It is stated by many that the diagnosis between the *Enteritidis* and the *Paratyphosus* B. can easily be made, by means of trustworthy sera for the respective organisms, but this is not our experience. We were not in a position to make a diagnosis although we obtained an *enteritidis*-serum from the Lister Institute, London, through the kindness of Dr Ledingham, and another *enteritidis*-serum and a *paratyphosus* B.-serum from the Institute of Infectious Diseases, Berlin, through the kindness of Professor Lentz.

An extract from our experimental tables (Table D) may serve as an example.

The *enteritidis*-serum from the Lister Institute was absolutely unable to differentiate between the strains of the *B. paratyphosus* B. and *B. enteritidis* which we had in hand, although these strains proved later to be what they were represented.

Very much the same result was given by the enteritidis- and paratyphosus B.-sera from the Institute of Infectious Diseases, Berlin, so that we could arrive at no definite conclusion as to the identity of the bacilli in the pies from agglutination with these sera, although the general result of the experimental evidence was in favour of the *B. paratyphosus* B.

TABLE D.

	Strains	Enteritidis-sera London	Enteritidis-sera Berlin	Paratyphosus B.-sera Berlin, Prof. Lentz
(1) Enteritidis. "Pr., Metropolitan Asylums Board"	... ... ... ...	1280	6400	5000
(2) Enteritidis. "Uhlenhuth, Kais. Gesundheitsamt, Berlin"	... ... ...	320	1600	100
(3) Paratyphosus B. "Pr., Metropolitan Asylums Board"	... ... ...	1280	3200	5000
(4) Paratyphosus B. "Rennes, Pasteur's Institute, Paris"	... ... ...	3200	800	5000
(5) Culture No. 1 as above	... ... ..	320	3200	5000
(6) Culture No. 9 a (direct from Pie 2)	...	160	3200	5000
(7) Culture No. 17 as above	...	80	1600	5000
(8) Culture No. 21 (from liver of fatal human case)	... ... ...	320	3200	5000
(9) Culture No. 4 (from heart blood of a guinea-pig injected with an emulsion of Pie 1)	...	640	1600	1600
(10) Culture No. 12 b (from heart blood of a guinea-pig injected with an emulsion of Pie 2)	...	640	1600	1250

We were, however, at last (beside the agglutination toxicity tests, etc.) enabled to make an absolutely certain diagnosis by the use of sera prepared by us with strains obtained from cases during the outbreak, as well as through the enteritidis- and paratyphosus B.-sera obtained from Professor Uhlenhuth (Kaiserlichens Gesundheitsamt, Berlin). The following Table serves to show this:—

TABLE E.

Strains	Prof. Uhlenhuth's sera		Sera prepared by us with strains	
	Enteritidis	Paratyphosus B.	No. 1	No. 17
Culture No. 1	...	125	8000	6400 3200
," No. 9 a	...	125	8000	3200 6400
," No. 17	...	125	8000	1600 12800
," No. 21	...	125	8000	3200 6400
Paratyphosus B. "Pr., Metr. Asylums Board"	...	125	8000	3200 6400
Paratyphosus B. "Rennes, Pasteur's Institute, Paris"	...	125	8000	3200 6400
Enteritidis. "Pr., Metr. Asylums Board"	...	4000	500	— —
Enteritidis. "Uhlenhuth, Kais. Gesundheitsamt, Berlin"	...	4000	250	— 100

The results of these experiments are absolutely clear : the organisms which caused the outbreak were *B. paratyphosus* B. The diagnostic difficulties which occurred in this case were due to the difference which exists in respect of agglutination binding and agglutination formation, *i.e.* the specific binding groups failed with the majority of our strains.

We should like in conclusion to draw attention to the practical importance of this investigation, which shows the bearing of the question of *Bacillus paratyphosus* B. carriers in their relationship to the preparation and sale of food. For milk this importance is already recognized, but for other foods the transmission of this bacillus through carriers is, as far as we are aware, not yet described. This case, therefore, is of great interest.

It is clear from a practical standpoint that persons who have passed through a paratyphoid illness should not be occupied in the food trade, unless repeated bacteriological examinations of their excreta have afforded negative results. Such preventive measures would not exclude, however, healthy bacillus carriers from causing outbreaks similar to the one here described.

ON THE NATURE OF THE CELLULAR ELEMENTS PRESENT  
IN MILK. PART III. THE MILK OF ANIMALS OTHER  
THAN THE COW.

FOR THE BRITISH DAIRY FARMERS' ASSOCIATION.

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HAVING arrived at the conclusions stated in our last report (Hewlett, Villar and Revis, 1910, p. 91), it seemed advisable to investigate the milk of animals other than the cow, to which hitherto we had confined our attention.

For this purpose we obtained samples of milk from the ass and goat, and also from the human subject. Naturally we were not able to carry out these investigations in the systematic manner we had hitherto employed, but such systematic treatment was at the same time scarcely necessary. These selected animals provide a field of enquiry very different from that which the cow presents. The ass may be taken as the type of an animal from which it is possible to obtain milk in but limited quantity and only so long as the foal is present. The lactation is in no way "artificial," if such a term may be used, and the animal itself is of a quiet, docile and stolid nature.

In the case of the goat we have an animal much more of the type of the cow, in that she will give milk over a fairly extended period

without the necessity of keeping the kid by her. The milk-producing power has not however been developed to such a degree as in the cow, at least in goats usually found in England, and certainly not in the case of those used in our experiments.

Milk production in the human subject is in type similar to that in the ass, but we have here to deal with a highly sensitive and nervous organization, which often affects lactation in a most profound manner.

It is most necessary to lay great stress on the fact that the milk-producing powers of the cow are the result of careful selection and breeding, so that both the udder itself and the process of lactation have become to a large extent artificial. The result is easily seen in the extremely sensitive nature of the cow's udder, making it react quickly and profoundly to external stimuli and rendering it very liable to damage and disease. It is for this reason that the cow's udder, in which the tendency to cell proliferation has been artificially encouraged by attempts to increase milk production, is liable to respond in a hyper-sensitive manner to minute causes, exactly in the way in which our experiments have indicated.

The relative position of the three specified animals is seen in a comparison of their body weight and average milk production, though it is of course extremely difficult to give either average weights or average milk yields in the case of any one of them.

	Body weight	Milk yield per day
Ass	450 lbs.	4 pints
Goat	100 „	2 „
Cow	1,100 „	24 „

Experiments were carried out exactly as in the case of cows; counts were made at frequent intervals and stained specimens were also prepared.

#### *The Ass.*

Counts of the cellular elements in the milk of this animal are much hindered by the constant presence of a white semi-crystalline deposit, which comes down on centrifugation. This deposit was examined and appeared to be of a protein nature, but no reason for its presence can be given, nor was the precise nature of it elucidated.

*Ass I.* First sample about 3 weeks after foaling.

Date	Cells per c.c.	Date	Cells per c.c.
23. iii. 10	32,000	14. iv. 10	168,000
2. iii. 10	38,000	21. iv. 10	55,000
17. iii. 10	198,000	27. iv. 10	436,000
24. iii. 10	910,000	5. v. 10	21,000
31. iii. 10	94,000	11. v. 10	66,000
6. iv. 10	444,000	Milk ceased.	

This ass showed greater variations and at times bigger cell counts than were observed in any of the other cases examined.

*Ass II.* First sample 4 weeks after foaling.

Date	Cells per c.c.	Date	Cells per c.c.
7. vi. 10	50,000	6. vii. 10	12,000
14. vi. 10	20,000	13. vii. 10	25,000
22. vi. 10	15,000	17. vii. 10	14,000
29. vi. 10	39,000		

The cell count was at all times small and very uniform.

*Ass III.* First sample 3 weeks after foaling.

Date	Cells per c.c.	Date	Cells per c.c.
26. vii. 10	12,000	27. ix. 10	20,000
9. viii. 10	7,000	19. x. 10	Deposit so large that no count was possible
16. viii. 10	12,000		
25. viii. 10	24,000	25. x. 10	10,000
30. viii. 10	16,000	9. xi. 10	26,000

The milk of this ass was distinguished by the white deposit referred to, being in greater quantity than in the case of any of the other asses. On one occasion (19. x. 10) the deposit was quite  $\frac{1}{8}$ th inch deep in the rotation tube, and precluded any hope of counting the cells.

*Ass IV.* First sample 3 days after foaling.

Date	Cells per c.c.	Date	Cells per c.c.
22. ix. 10	16,000	7. x. 10	42,000
26. ix. 10	16,000	11. x. 10	23,000
30. ix. 10	44,000	14. x. 10	22,000
4. x. 10	24,000	25. x. 10	10,000

On account of the recent foaling, samples were examined every fourth day, but no effect at all on the count was observed. On several occasions, notably from the first to the fourth sample, much of the heavy white deposit was present. From 7. x. 10 nearly all the cells were large in size and resembled the so-called "colostral bodies." The cell count was always small and fairly constant.

A certain number of stained preparations were examined with the following results:

No. of animal	Date	Nature of cells present
<i>Ass I</i>	23. II. 10	Mostly large uni-nuclears and multi-nuclears.
	" 14. III. 10	Cells very scanty—a few vacuolated cells and small uni-nuclears.
	" 24. III. 10	Large number of multi-nuclears, with a few large and small uni-nuclears and vacuolated cells.
	" 31. III. 10	Cells scanty, a few large and small uni-nuclears, multi-nuclears and vacuolated cells.
	" 7. IV. 10	Cells scanty, as in last specimen.
	" 20. IV. 10	As in last specimen.
<i>Ass II</i>	17. VI. 10	Cells scanty, mostly large uni-nuclears.
	" 22. VI. 10	Very few cells. A few large and small uni-nuclears.
	" 29. VI. 10	Very few cells. Mostly small uni-nuclears, with some multi-nuclears and a few large uni-nuclears.
	" 6. VII. 10	Cells practically absent.
	" 12. VII. 10	Cells very scanty; a few small uni-nuclears.
<i>Ass III</i>	—	The slides contained so few cells that the examination was of no real value.

All these samples of asses' milk were distinguished by low cell counts and the cells themselves stained with difficulty. That is quite in accordance with our conclusions as it is evident that these cells are in such cases only cast off slowly and are consequently in a degenerate condition, hence their poor staining capacity and indefiniteness.

#### *The Goat.*

For the samples of goats' milk we are indebted to the kindness of W. Edmunds, Esq., M.D., who having three goats at the Brown Institution allowed us to have samples weekly from these animals.

The description of the goats is as follows:

*Goat I.* Normal Anglo-Nubian. Born March 23rd, 1906. Kidded about March 30th, 1910. Weight  $47\frac{1}{2}$  kilos.

*Goat II.* English. Born in 1903, or 1904. In November 1906, the thyroid gland was excised. Kidded April 18th, 1910. Weight  $44\frac{1}{2}$  kilos.

*Goat III.* Born April 1904. Both thyroids excised January 10th, 1907. Kidded March 21st, 1910. Weight 39 kilos.

All these goats were in splendid condition and quite healthy with the exception of a temporary indisposition in the case of Goat II. (See below.)

*Goat I.*

Date	No. of cells per c.c.	Date	No. of cells per c.c.
21. vi. 10	182,000	12. vii. 10	110,000
28. vi. 10	152,000	17. vii. 10	143,000
6. vii. 10	194,000	26. vii. 10	209,000

The cell count was very regular and low, but was interfered with by the presence of much débris and the fact that many of the cells were very small.

The microscopical examination of five slides gave the following results :

Date	Nature of cells present	
21. vi. 10	Many vacuolated cells. Other cells mainly small uni-nuclears, some of the normoblastic type.	
28. vi. 10	As in the last specimen.	
6. vii. 10	Mostly small uni-nuclears and some vacuolated cells.	
12. vii. 10	Large and small uni-nuclears.	
17. vii. 10	Mostly small uni-nuclears, with a few large uni-nuclears and vacuolated cells.	

*Goat II.*

Date	No. of cells per c.c.	Date	No. of cells per c.c.
21. vi. 10	2,230,000	12. vii. 10	382,000
28. vi. 10	7,510,000	17. vii. 10	218,000
6. vii. 10	280,000	26. vii. 10	286,000

The very large cell count of the first two samples is noteworthy and cause for it was sought. The goat was perfectly healthy and the count was evidently not normal, as it fell the following week to a lower figure and remained low. On careful inquiry it was found that this goat had arrived after a railway journey at the Brown Institution on 8. vi. 10 in rather poor condition and had had diarrhoea till the 13th. Since that date she improved rapidly in condition and the illness could only be ascribed to the railway journey. This is the only cause that can be assigned for the increased cell count and according to our views is by no means improbable, and would illustrate again the effect of external causes on cell proliferation in the udder.

Three slides were stained and examined.

Date	Nature of cells	
21. vi. 10	Chiefly small uni-nuclears, many of the normoblastic type, but many large uni-nuclears and multi-nuclears and a few vacuolated cells.	
12. vii. 10	Mostly small uni-nuclears.	
17. vii. 10	As in the first specimen.	

*Goat III.*

Date	No. of cells per c.c.	Date	No. of cells per c.c.
12. vii. 10	311,000	2. viii. 10	316,000
17. vii. 10	521,000	9. viii. 10	538,000
26. vii. 10	184,000	16. viii. 10	680,000

The cell count is, as will be seen, fairly uniform, and calls for no comment. Stained preparations were practically identical in nature, the cells consisting mostly of multi-nuclears with some small uni-nuclears (many of the normoblastic type) and a few large uni-nuclears.

The milk of these three goats showed exactly the same types of cells as have been met with in the case of other animals. The fairly uniform count (with the exception of the two samples of Goat II already referred to) is probably the result of the easy and regular life of the animals. It is to be noted that the excision of the thyroid gland has no perceptible effect on the cell count.

*Human Milk.*

By the kindness of the Medical Officer of the Queen Charlotte Hospital, Marylebone Road, a few samples of human milk were obtained and examined. It was not, in the nature of the case, possible to obtain successive samples, and each is dealt with separately.

*Sample I*, taken 11. iv. 10, seven days after birth. First child. Breasts normal and progress satisfactory.

Cells per c.c. 2,960,000. Stained preparations showed that a large number of the cells stained poorly and were therefore rather ill-defined. The majority of the cells were small uni-nuclears and multi-nuclears, with a few large uni-nuclears and vacuolated cells.

*Sample II*, taken 7. vii. 10, five days after birth. First child. Breasts and lactation normal.

Cells per c.c. 252,000, but the cells were in clumps and probably the number is too low. Total deposit large. The cells were chiefly large uni-nuclears, many having semi-lunar or horse-shoe nuclei. Some small uni-nuclears, but practically no multi-nuclears.

*Sample III*, taken 8. vii. 10, six days after birth. First child. Breasts and lactation normal.

Cells per c.c. 2,640,000. Deposit large and the count was rendered difficult by the presence of a large number of very small indefinite cells, which are not included.

The stained preparations showed that many cells were ill-defined, but almost all were large and small uni-nuclears. Many large uni-nuclears had semi-lunar or horse-shoe nuclei. Practically no multi-nuclears.

*Sample IV*, taken 7. vii. 10, two days after birth. Fifth child. Breasts and lactation normal. Total deposit large.

Cells per c.c. 7,440,000. The cells were large and well-defined and in marked contrast to the foregoing samples. They were of all types, large and small uni-nuclears, multi-nuclears and vacuolated cells and some eosinophiles.

*Sample V*, taken 11. vii. 10, three days after birth. Number of children not stated. Breasts and lactation normal. Milk very yellow.

Cells per c.c. 563,000. Again the cells were well-defined and consisted of large and small uni-nuclears and some multi-nuclears and many vacuolated cells.

*Sample VI*, taken 11. vii. 10, six days after birth. Fifth child. Breasts and lactation normal. The milk which was small in quantity was very thin and watery and there was a deposit of casein. A reliable count was impossible, but there were a large number of cells which in stained preparations were similar to those of Sample V.

The number of samples is too small for any generalisations, but it must be noted that in the three samples obtained from multipara, the cells were much better defined and more varied in nature than in the case of the other three samples from primipara. The cells themselves were of quite the same nature as in other milks. One cannot pass by the very high cell counts obtained in one or two cases of these *human* milks without a thought of the objection, so often raised in the case of cows' milk, that the presence of "pus" in milk, if not actually harmful, is "sentimentally" repulsive!

#### SUMMARY.

The general consideration of these results only tends to confirm the conclusions already arrived at. A uniform type of life evidently tends to a fairly uniform excretion of tissue cells from the udder. The case of Goat II also emphasises the effect of outside causes in increasing temporarily this excretion, while some of the samples of human milk show plainly that very high cell counts are not by any means necessarily connected with any diseased or disordered condition of the mammary gland.

We again emphasise the view we have already stated, viz. that in the cow the udder must be looked upon as an organ which has by breeding and selection been brought to an artificial condition of milk secretion and that this has been accompanied by a stimulation of the tissues to cell proliferation and that this proliferation may quite easily be caused to become abnormally great, leading to the appearance of an increased number of cells in the secretion. In support of this view we lay great stress on the fact constantly noticed by us, viz. that when the cell count is high for *any* reason, the cells themselves are always well-defined, showing little signs of degeneration, and also stain in a much more characteristic and definite manner, a fact which is difficult to explain if they are to be considered to be blood elements.

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## THE PRECIPITIN, COMPLEMENTBINDING, AND ANTI-OPSONIC TESTS IN TUBERCULOUS AND NORMAL CATTLE.

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THE recognition of latent tuberculosis, difficult as it often is in the human subject, is of necessity more difficult in the case of cattle. It is especially in early disease that the serum reactions prove often most useful as an aid to diagnosis, but in cattle a proof is also often desired that no pronounced disease is present, because it is by no means of rare occurrence that an apparently fat and healthy animal must be rejected after slaughter.

We have in tuberculosis a large number of immunity reactions, some of which are associated with the early stages of the disease, and others which are characteristic only of the end. It is well to keep this clearly in view, otherwise too much is apt to be demanded of a particular test, with consequent disappointment. It is doubtful if any single test exists which is absolutely characteristic of tuberculosis, and in all stages of the disease.

Even the tuberculin injection test, which is generally held to give the best results (Szaboky (1909, p. 274), for example, found that it gave the greatest number of positive results of 11 different serum and bacteriological tests applied by him), is yet regarded by many practical men as unreliable. The percentage of slaughtered cattle showing macroscopic tubercle varies, according to Ostertag, between 6·34 and 45 %. The percentage of cattle reacting to the tuberculin test is however infinitely higher. Siedamgrotsky tested 259 cattle and obtained a typical reaction in 76 %. Bang and Nocard in Denmark

and France found 2/3 to 3/4 of animals examined affected. Similar results have been obtained in other countries. It is a well-known fact that animals reacting to tuberculin have been slaughtered, and no macroscopic evidence of tubercle has been found upon examination. Undoubtedly the explanation of this depends upon the delicacy of the test, so that by its means minimal traces of disease are made evident. On the other hand the tuberculin reaction may entirely fail in the later stages of the disease. Animals suffering from advanced tuberculosis, which after slaughter have been found to be riddled with the disease, have often failed to react to injections of tuberculin. The cattle-dealer who trusted to the reaction to give reliable information as to whether his meat would be saleable or not naturally lost faith in the test. But in spite of this the reaction as an indication of the presence of early or latent tuberculosis remains of the greatest value.

Many serum reactions in tuberculosis, like the tuberculin injection test, seem to have the same drawback, *i.e.* that a percentage of apparently normal sera also react. A reaction regarded by many as characteristic rather of early than of late disease, the agglutinin reaction, introduced by Arloing (1898, p. 1398), was given in the hands of that author by 22% of normal human persons examined, and 10 out of 50 normal cattle (1901, p. 712). Beck and Rabinowitch (1901, p. 145), on the other hand, obtained the reaction in practically all slaughtered cattle examined whether tuberculous or not.

Whether the agglutinin and precipitin tests depend upon the same immune substance is still under discussion in the case of other diseases. In the case of tubercle there is a somewhat strong probability that the two are identical. The agglutination tests as described by Arloing and Koch (1901, p. 821), were carried out with serum in somewhat high concentrations, 1:5, 1:10, 1:20. The precipitation test as I have used it (1910, p. 88), was carried out with human serum diluted 1:42, and apparently at such dilutions fewer normal sera react, because of 301 normal sera only 12.3% gave a decided precipitate, while of 381 tuberculous sera, 46.7% reacted strongly. The greatest percentage of positive results given by these human sera were yielded by chronic cases. Advanced sera were more often negative than early sera, and nearly three times as often negative as chronic sera. The precipitin reaction in the case of human sera appears to be especially characteristic of chronic tuberculosis.

A tubercle reaction which I examined at the same time as the precipitin test in human serum was the complementbinding test for

the presence of antibody in human serum; this I found more commonly present in advanced disease, and often in cases where the precipitin test failed. A similar experience was obtained with Marmorek's test (1911) for the antigen in urine by means of complementbinding. Here again the test appeared more characteristic of advanced disease.

Another reaction, which Fornet and I (1909, p. 138) believed to be characteristic rather of advanced sera, was called by us the antiopsonic test as it depends upon the presence of an antagonism in heated serum to the action of fresh opsonin.

An antagonism between heated serum and fresh has been observed in the case of various immune reactions. This antagonism was described first by Neisser and Doering (1901, p. 595), in the case of complement in disease, and studied by v. Bergmann and Savini (1907, p. 817), Eva Hoffmann (1907, p. 704), and others. Camus and Payniez (1901, p. 730) have met with it in the case of simple isolysins apart from complement, and Welsh and Chapman (1907, p. 465) have described an antiprecipitin which as it is produced by a temperature above that required for inactivation they do not regard as merely a precipitoid. In the case of ferments Cramer and Bearn (1906, p. 36), Pollak (1905, p. 95), and Schwarz (1905, p. 524), have had similar experience.

Fornet and I (1907) met with this phenomenon in the case of opsonins and as already stated we ascribed it to the appearance of an anti-opsonin. The same result has been since then observed by various authors especially where concentrated inactive serum has been used (Rosenthal (1909), Pribram (1910, p. 1131)), but they hesitate to adopt the theory of an actual anti-opsonin. Similar results have been obtained by Hektoen and Ruediger (1905, p. 128), who found absorbed opsonin inhibitive after heating, and by Haetjens (1907, p. 560), who after absorbing bacteria with heated serum found that they were not opsonised by fresh serum, which was perfectly able to opsonise untreated bacteria.

Fornet and I were disposed to lay some stress upon the importance of this phenomenon on account of the fact that this antagonistic substance was produced not only by heat but also in the neighbourhood of bacteria, and even of artificial membranes. The inactivation of opsonin in the presence of reed membranes has recently been confirmed by Pribram (1910, p. 1131), while Ledingham (1907, p. 482) has noticed the appearance of an antagonistic influence in serum through which bacteria had been passed, which he ascribed to bacterial products. The inactivation of ferments in contact with artificial membranes has been

noticed by Slosse and Limbosch (1909, p. 417), and myself (1910, p. 301), a process which was not explained by simple absorption, and which was accompanied by the appearance of antiferments. The production of antiferments by natural membranes with a view to their own protection has been described by Weinland (1902, p. 45), in the case of the stomach and intestines, while Kantorowicz (1909, p. 897) has described an inactivation of trypsin in the membranes of bacteria. Again, Pfeiffer and Friedberger (1905, p. 1145), and Bail and Kikuchi (1905, p. 275), have noticed an analogous behaviour on the part of bacteria against amboceptor, and Weil (1905, p. 291) has described the same in the case of agglutinin. On account of the fact that artificial membranes can powerfully exert this influence at least upon complement, opsonin, and certain ferments, the effect depending greatly upon the physical condition of the membranes, transparency etc., the action of such membranes as a whole may be taken as due in part to a physical property of membranes upon absorbed substances. The possibility of the inactivation of immune substances and the actual production of antagonistic agents by bacterial membranes in the human body must not be lost sight of, as it seems to present a weakness in the defences of the organism.

It appears highly probable that the absorption of opsonin by bacteria in the absence of leucocytes is unnatural, and if occurring in the animal body, unfortunate. A large number of authors at the present time, for example Neufeld (1904, p. 1458), and his collaborators, Hektoen (1909, p. 66), Rosenow (1906, p. 683), Fornet and myself (1909, p. 156), regard the action of phagocytic agents in the serum as an agglutination of bacteria to leucocytes. This view is held partly because of the great rapidity with which phagocytosis takes place, but mainly because the clumping of masses of leucocytes and bacteria together, which is an invariable accompaniment of phagocytosis where the process is not prevented by the presence of large numbers of red cells, has also been observed with dead leucocytes.

Hektoen (1906, p. 19), Weil and Tsuda (1907, p. 1038), Levy and Fornet (1906, p. 1039), and Fornet and myself (1909, p. 166), have noticed that culture filtrates hinder phagocytosis by acting against the leucocytes.

It appears likely that while the bacterial products keep the leucocytes at bay and counteract agglutination an unnatural absorption of opsonic substances is taking place followed by an inactivation of opsonin and production of antiopsonin in the bacterial membrane. The partial

disappearance of opsonin in the bacterial wall, after absorption in vitro, has been pointed out by various authors, Dean (1905, p. 506), Meyer (1908, p. 951), Centanni (1908, p. 140), Sellards (1908, p. 308), Hektoen (1909, p. 74), and Fornet and myself (1909, p. 147).

The antiopsonin is most easily and rapidly produced by heat and is therefore best measured in heated serum.

In the following experiments I have examined the sera of 408 cattle (including 30 tuberculous) for the precipitin reaction, both with bovine and human tubercle extract, of 80 cows (including 18 tuberculous) with the complementbinding test for antibody, and 118 cows (including 20 tuberculous) with the antiopsonic test, with a view to testing their value as diagnostics of the presence of tuberculous disease in cattle, and incidentally to note the number of normal animals reacting.

#### *The precipitin test.*

The following results were obtained with the precipitin test, the expression normal being used to denote animals in which no macroscopic tubercle was found after slaughter.

*Method.* The method employed was similar to that which I have already described in a former paper (1910, p. 88). Bovine and human tubercle bacilli were ground with distilled water and the mixture shaken and kept at 37° for 36 hours, sufficient NaCl and phenol being added to render the concentration of NaCl 0·85 %, and of phenol 0·5 %. The extract was then filtered through a Berkfeld filter. One drop of serum was placed in a narrow tube and diluted with 20 drops of physiological salt solution to 1/21. Seven drops of diluted serum were removed into a second tube, and seven into a third. This dilution of the serum to 1/21 was not critical in any way. The dilution was chosen simply because while not too weak it yet gave a convenient amount of fluid in the tube (which was of the narrowest size) for a precipitate to be conveniently examined.

An equal quantity of bovine tubercle extract was added to the first tube, of human tubercle extract to the second, while the third (control) tube received a solution containing 0·85 % NaCl and 0·5 % phenol alone. Every tube therefore contained phenol. As it was found that bovine serum is less liable to precipitate with phenol alone than is the case with human serum, controls containing tubercle extract without phenol were not used, only controls containing phenol without tubercle extract being employed.

The tubes which had been filled with sterile precautions were stoppered with sterile wads and placed at 37° for 12 hours, after which they were examined for precipitates.

*Normal Cows' Serum.*

No. of animals tested	Tested with	Result				Percentage	
		Negative	Doubtful	Positive	Strong	Positive	Negative
180	Bovine extract in 0·85 % NaCl and 0·5 % phenol	119	9	30	22	29	66·1
180	Human extract in NaCl, etc.	134	7	24	15	21·6	74·4
180	0·85 % NaCl and 0·5 % phenol alone	172	5	3	—	1·66	95·5

Fourteen of these 180 sera reacted to bovine tubercle extract alone not to human, and 4 reacted to human tubercle extract but not to bovine.

*Normal Bullocks' Serum.*

No. of animals tested	Tested with	Result				Percentage	
		Negative	Doubtful	Positive	Strong	Positive	Negative
198	Bovine extract in 0·85 % NaCl and 0·5 % phenol	151	14	29	4	16·66	76·2
198	Human extract in 0·85 % NaCl and 0·5 % phenol	164	11	21	2	11·6	82·82
198	0·85 % NaCl and 0·5 % phenol	184	11	2	1	1·5	92·98

Ten of these 198 sera reacted alone to the bovine tubercle extract, one very strongly, without showing any trace of precipitate with human tubercle extract. Two reacted alone to human extract without responding to bovine.

From the above results it will be noticed that,

- (1) a somewhat large percentage of apparently normal animals reacted,
- (2) a larger number of cows reacted than of bullocks,
- (3) that while the majority reacting did so to both human and bovine tubercle extract, a certain number reacted only to one, generally the bovine extract. (It may be mentioned that precipitates to the bovine extract were generally stronger than to the human.)
- (4) the precipitates to 0·5 % phenol in physiological salt solution were extremely few in number. (In this respect the serum of cattle differs greatly from human serum, which precipitates almost as frequently

with phenol as with tubercle extract which contains no phenol, both precipitates occurring in the same serum.) The reaction is correspondingly more reliable.

I should like to mention that Mr Thomson, the Veterinary Inspector who was so kind as to collect the sera and examine the carcasses, gave the specimens to me in batches without special information about them. He was often able to notice in the results afterwards that reacting animals had come from the same source, where they must have been exposed to the same conditions. On this account and also because of the fact that more cows react, which are well known to be more exposed to infection, it is very probable that we have here to deal with cases of early tuberculosis so extremely slight as to be unrecognisable at the ordinary examination after slaughter, rather than with an inaccuracy of the method.

#### *Tuberculous cattle.*

The only tuberculous sera which I was able to obtain were extremely small in number, only 30. The majority of these were from advanced cases.

No. of animals tested	Tested with	Result				Percentage	
		Negative	Doubtful	Positive	Strong	Positive	Negative
30	Bovine extract in 0·85 % NaCl and 0·5 % phenol	8	6	10	6	53·3	26·6
30	Human extract in 0·85 % NaCl and 0·5 % phenol	16	4	7	3	33·3	53·3
30	0·85 % NaCl and 0·5 % phenol	28	2	—	—	Nil	93·3

One case acted very strongly to human tubercle extract but not at all to bovine, and one which acted to both did so more strongly to human than bovine. Eight were positive to bovine extract but not human. The bovine precipitates were usually the stronger, but the results obtained were on the whole somewhat weak, and the percentage of positive results fewer than might have been expected. This was probably due to the fact that the sera were almost all from cases of advanced disease.

One case might be described as of interest. A cow was found to be spitting up a quantity of sputum in which tubercle bacilli were discovered. The animal was condemned and slaughtered. After slaughter no evidence of tubercle could at first be detected, the organs appearing healthy. Congestion of the trachea was then observed and tubercle

bacilli were found microscopically in the mucous membrane. The serum of this animal precipitated very strongly indeed to bovine tubercle extract, but not at all to human tubercle extract. This was the only case of early tuberculosis examined.

*The complementbinding test.*

*Method.* After the tubes prepared as above described for the precipitin reaction had been examined and any precipitates noted, complement was added in an amount sufficient to haemolyse 1·0 c.c. of 4% sheep's corpuscles, i.e. 0·1 c.c. of guinea-pig serum. The tubes were then placed in the incubator again for an hour and at the end of this time 0·5 c.c. of amoceptor (immune rabbit's serum) and 1·0 c.c. of 4% sheep's corpuscles added. The mixture was again placed at 37° for two hours.

Eighty sera were tested in this way, including 18 tuberculous.

*Normal Cows' Serum.*

No. of animals	Tested with	Result :—Haemolysis			Percentage	
		Complete	Partial	Absent or trace	Binding	Not binding
62	Bovine extract in 0·85% NaCl and 0·5% phenol	50	9	3	18·35	80·64
62	Human extract in 0·85% NaCl and 0·5% phenol	50	10	2	19·35	80·64
45	0·85% NaCl and 0·5% phenol	45	—	—	—	100

Eight normal sera which did not precipitate with human tubercle extract (12·9%), bound complement in its presence (of these sera 5 had however precipitated to bovine extract). On the other hand 3 sera which had precipitated with bovine and human tubercle extract failed to bind complement.

*Tuberculous Cows' Serum.*

No. of animals	Tested with	Result :—Haemolysis			Percentage	
		Complete	Partial	Absent or trace	Binding	Not binding
18	Bovine extract in 0·85% NaCl and 0·5% phenol	2	2	14	88·88	11·11
18	Human extract in 0·85% NaCl and 0·5% phenol	5	4	9	72·22	27·77
18	0·85% NaCl and 0·5% phenol	18	—	—	—	100

Ten tuberculous sera (55 %) which had not precipitated with human tubercle extract, bound complement in its presence (of these sera 3 had however precipitated with bovine extract).

*The antiopsonic test.*

*Method.* Wright's method was employed. The leucocytes were from human blood, the bacillary emulsion from Allen and Hanbury's moist preparation.

The middle point between two estimations of the same normal bovine serum, measured at the same time, was taken as 1. A group of sera under investigation were heated for half an hour at 58° to 60°, and were then tested as to their power of reducing the opsonic value of this fresh normal serum.

It was found that while with 1 part of fresh normal serum, 1 part of physiological salt solution, 1 part of leucocytic and 1 part of bacillary emulsion the index stood at 1, when the physiological salt solution was replaced by heated bovine serum the resulting opsonic value varied between 0·1 and 1·27, but was generally lower than the control 1. By using 1 as a standard it was made possible to compare all these different estimations made on different days with different sera.

Altogether 118 heated cows' sera were tested including 20 tuberculous.

*Normal Cows' Serum.*

Number tested 98.

Index changed from 1·0 to between	No. of sera	Percentage
0·1 and 0·2	1	
0·2 „ 0·3	3	
0·3 „ 0·4	8	18·3
0·4 „ 0·5	6	
0·5 „ 0·6	5	
0·6 „ 0·7	14	
0·7 „ 0·8	17	
0·8 „ 1·0	10	
1·0 „ 1·1	17	
1·1 „ 1·2	6	24·4
1·2 „ 1·3	1	
		75·5

In 75·5 % of cases the opsonic value of normal bovine serum was lowered by the heated serum. In 18·3 % of cases it was reduced to below one-half its former value.

Of these 18 sera which were so powerfully antagonistic 3 reacted slightly to the precipitin test, none bound complement. As a negative precipitin test points either to advanced disease or to absence from disease, and in these cases certainly no marked disease was present, the extreme antagonistic power of these sera indicates rather an inborn susceptibility, a flaw in the protective mechanism rather than necessarily a proof of disease.

*Tuberculous Cows' Serum.*

Number tested 20.

Index changed from 1.0 to between	No. of sera	Percentage
0.1 and 0.2	2	
0.2 , , 0.3	5	
0.3 , , 0.4	3	
0.4 , , 0.5	3	
0.5 , , 0.6	1	
0.8 , , 0.9	1	
0.9 , , 1.0	2	
1.0 , , 1.1	2	15
1.1 , , 1.2	1	

In 85% of cases the opsonic value of fresh serum was lowered, in 65% of cases to below one-half its former value. The difference between normal and tuberculous serum is striking. All the serum which did not lower the opsonic value, precipitated and bound complement, while of the 13 which lowered the value by more than a half, as many as 9 did not precipitate.

**CONCLUSIONS.**

Advanced tuberculous bovine serum, like human, does not respond very well to the precipitin test. On the other hand a certain percentage of animals without any macroscopic evidence of tubercle, do react. On this account the reaction, although undoubtedly valuable as a prophylactic, does not afford very reliable information as to the condition of an animal about to be slaughtered for meat. Combined with the complementbinding test which appears to be more characteristic of advanced than of early disease (the precipitin reaction belonging rather to the early and intermediate stages), it should prove of considerable value. It is interesting that bovine tuberculous serum does not precipitate with 0.5% phenol, in the same way as human tuberculous serum.

The antiopsonic reaction can hardly be said to be characteristic of tuberculous disease. It represents apparently a flaw in the normal protective mechanism, and if especially present in advanced tuberculous sera is probably only so because the individuals possessing this pre-antiopsonin were rendered thereby more susceptible to the progress of the disease.

If the precipitin reaction is on the whole perhaps prognostically more favourable, the antiopsonic reaction is probably prognostically unfavourable.

I should like to express my thanks to Mr Thomson, Veterinary Inspector, Edinburgh Slaughterhouse, for his kindness and the great trouble he has taken in collecting the specimens of blood, and examining the carcases.

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## THE QUANTITATIVE ESTIMATION OF INDOLE.

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DURING my work in Yucatán (tropical Mexico) I observed that we nearly always found by the current analysis of urine, a well pronounced indican reaction, certainly in more than 80% of the cases examined; and frequently the blue colour obtained by the Obermeyer or Jaffé reaction was so strong that one would have been inclined, if in Europe, to postulate the presence of intestinal obstruction or some serious intestinal disorder. Experience soon taught us however that the reaction had not the same significance in Yucatán and probably in other tropical countries as it possesses elsewhere. With the object of studying the underlying causes I suggested to one of my assistants an investigation of the indole-producing power of *B. coli* isolated from the faeces of different individuals. The results which have been published in a dissertation (Hernandez, 1908) were naturally very incomplete but the work led me to realise the importance of a quantitative indole-determination, and, as I did not find described in the literature any method suitable for our purpose, I proceeded to work out a method for myself. I used the Salkowski test, with nitrite of potassium, sulphuric acid, and extraction with amyl-alcohol, preparing a series of tubes containing determined quantities of indole in progressive concentrations. The indole I distilled from bacterial cultures. The scale kept well, having been preserved for three years in the dark and only brought into the light when in use.

Working at the Lister Institute, I desired to make a similar scale, using the more sensitive indole test with paradimethylaminobenzaldehyde and potassium persulphate, known as the Ehrlich test. Considerable difficulties were however encountered, as the colour soon faded, when

the tubes were preserved for a short time, and it was only after many attempts that I found a useful method.

Very little has been published regarding the quantitative estimation of indole. The most exact method is probably Marshall's (1907), but as it necessitates in each case first a distillation and afterwards a rather laborious colorimetric process it is not likely to enter largely into ordinary bacteriological routine. The same may be said of the methods of Peckham (1897), and Herter and Foster (1906). They are too complicated for daily work, and a method is needed which does not involve the use of distillation or other apparatus and which can, if possible, be completed in a few minutes. This object would apparently be attained by Crossonini (1910), who uses a direct colorimetric comparison, his scale consisting simply of a series of tubes in which he has obtained Ehrlich's reaction. Here again it would however be necessary to make a new scale almost every day, as the colour, especially in the weaker indole-solutions, fades very quickly when preserved in this manner, even if the tubes are kept in darkness. Crossonini does not use extraction and does not start with very weak solutions and accordingly the method presently to be described is not only much more reliable but also more sensitive and convenient.

Some few principles may be laid down for a useful reaction and estimation.

The first principle must be that the reaction shall demonstrate small quantities of indole, and for that purpose it is necessary to extract the indole either with amyl-alcohol or with chloroform, as the colouring substance can be concentrated in this manner in a smaller volume of liquid and it will moreover be more easily observable, being transferred from a yellow to a colourless medium. For a qualitative test it may be preferable to use only a very small quantity of 1 or 2 c.c. but in a comparative scale it is evidently necessary to always extract with the same quantity of liquid which must not be too small, because variations would not show well, nor too large, otherwise faint reactions would pass unobserved. I have found that a quantity of 5 c.c. suits the purpose very well. By this method not only a dilution of one to a million gives a distinct reaction, as generally stated, but also the presence of indole in a proportion of one to ten millions may easily be demonstrated.

The second essential must be that the standard tubes may be kept for a very long time without alteration, that is to say without fading of the colour produced. As the fading seems to depend principally or entirely on the progress of the oxidizing process beyond the stage which is

necessary for the reaction, the object must be to exclude the possibility of a continued oxidation.

The continuance of this process is caused by the presence of the potassium persulphate.

Accordingly when we separate the coloured substance, after extraction, from the original liquid in which the reaction is produced, we eliminate the principal source of alteration. Simple contact with the air may also produce a certain amount of fading although this occurs after a much longer lapse of time; this fading might be prevented by keeping the extracts *in vacuo* or by substituting another gas for the atmospheric air, but a much simpler method is to preserve the extract below a liquid, such as sterile physiological salt solution which is indifferent by itself and which excludes the air to a sufficient degree. Amyl-alcohol is therefore unsuited for the purpose, but chloroform may be employed as the extracting liquid. I have not been able to find a liquid of less specific gravity than amyl-alcohol which does not mix with it. Moreover, as in the case of a very weak indole solution, the oxidation may progress very rapidly when the ordinary saturated potassium persulphate solution is employed, I prefer to use only a 1% solution, since it gives more trustworthy results. Using a weak solution the reaction attains its greatest intensity somewhat later, but it occurs nevertheless in the low numbers of the scale within half an hour. With higher concentrations of indole a few hours are required for the colour to attain its full intensity and the colour undergoes no noticeable alteration during several hours.

Thirdly it must be easy to distinguish between the different numbers of the scale and therefore the difference in concentration between any two following numbers must be in proportion to the absolute concentration. However, for various reasons, it would not be practical to use a geometric progression. I have therefore used a scale which runs from 1 to 100 but actually employing continuous numbers from one to ten only and hereafter omitting intermediate tubes as the concentrations mount higher.

Taking into consideration these principles I have developed the following technique. 5 centigrams of pure indole (Merck) are dissolved in a few c.c. of absolute alcohol, adding subsequently distilled water up to 500 c.c., making a 1:10,000 solution. This represents No. 100 of the scale.

Of this solution 1 c.c. is added to 99 c.c. of water, which represents a solution of 1:1,000,000 or No. 1 of the scale and in a corresponding

manner the Nos. 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 25, 30, 40, 50, 60, 70, 80, 90, are prepared so that the scale consists of 24 numbers. Of each solution 10 c.c. are introduced into a series of test-tubes, all of approximately the same diameter and rather wide. To each tube are added 5 c.c. of the ordinary Ehrlich's solution (paradimethylamidobenz-aldehyde 4, alcohol (96 %) 380, conc. hydrochloric acid 80) and 5 c.c. of a 1 % solution of potassium persulphate. When the red colour has reached its greatest intensity, 5 c.c. of chloroform are added and the whole is well mixed without shaking violently. The chloroform extracts nearly all the colouring substance and collects in the bottom of the tube so that there remains above only a very faintly stained liquid. By means of a burette the chloroform is separated into other tubes, which subsequently are nearly filled with sterile salt solution (0·85 %) and closed with cotton wool and paraffin or with rubber caps. When not in use the scale should preferably be kept in darkness, although so far as I have observed, exposure to light does not seem to have any deteriorating influence.

The difference between any two tubes in sequence is always sufficiently pronounced, so that it is easy to make a quick comparative colorimetric determination each time an indole reaction is made. For the ordinary reactions the saturated or the 1 % potassium persulphate solution may be used.

Several of the standard tubes have been kept for more than a month, being daily exposed to the daylight for some time, without any alteration.

By means of this scale a considerable number of indole determinations in different cultures have been made, which will be published at a later date.

I wish to express my gratitude to Dr C. J. Martin, F.R.S., Director of the Lister Institute of Preventive Medicine, for his kind permission to work at the Institute, and to Dr Ledingham for the interest with which he has followed the investigations.

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THE FLEAS COMMON ON RATS IN DIFFERENT PARTS  
OF THE WORLD AND THE READINESS WITH  
WHICH THEY BITE MAN.

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(With Plate II.)

THE appetite for mankind of the fleas which infest rats in different parts of the world has become a question of epidemiological importance in view of the conclusion now generally accepted, that in the present pandemic these parasites play the principal part in the transmission of plague from rat to rat and from rat to man.

Whether rat fleas bite man has been much controverted, ever since Ogata (1896) and Simond (1898) put forth the theory that bubonic plague was conveyed by infected fleas. The difference of opinion is to some extent due to want of recognition of the fact that rats in different parts of the world, and also when living under varying conditions in the same part of the world, may harbour fleas belonging to different species. After making due allowance for this source of error, however, the fact remains that two observers experimenting with the same species of fleas have, not infrequently, come to opposite conclusions. It is therefore clear that the number of observations recorded and the precise conditions under which the experiments were made must be taken into account.

It is not necessary to enumerate all the species of flea which have, on occasion, been captured off rats, because this has already been done by Tiraboschi (1904), Shipley (1908) and quite recently by Rothschild (1910). From the point of view of epidemiology, it is only important to know, what fleas infest rats in different parts of the world and whether these fleas bite man with any readiness.

*Fleas commonly infesting rats.*

Knowledge on this point is incomplete. It is only recently that a large number of rats has been examined with this object and so far comparatively few localities have been studied. The occurrence of plague in a locality has alone provided a sufficient excuse for taking a census of the fleas infesting the rats.

As far as at present known the fleas commonly infesting both *Mus rattus* and *Mus decumanus* are :

1. *Xenopsylla cheopis* (Rothschild).  
Synon. *Pulex cheopis* Rothschild, 1903.  
*Pulex pallidus* Tidswell, 1903.  
*Pulex brasiliensis* Baker, 1904.  
*Pulex murinus* Tiraboschi, 1904.  
*Pulex philippinensis* Herzog, 1904.  
*Loemopsylla cheopis* (Rothsch.) Rothschild 1908.
2. *Ceratophyllus fasciatus* (Bosc.).
3. *Ceratophyllus anisus* Rothschild, 1907.
4. *Ctenopsylla musculi* (Dugès).
5. *Ctenophthalmus agyrtes* (Heller).

The dog flea (*Ctenocephalus canis*), the cat flea (*Ctenocephalus felis*), the fowl flea (*Echidnophaga gallinacea*) and the human flea (*Pulex irritans*) are also sometimes found upon rats, but they appear to be only occasional visitors. In certain localities, however, they have formed quite a considerable proportion of the fleas captured. Tiraboschi (1904, p. 259) states that in several regions of Italy as many as 25% to 30% of the fleas on rats were dog and cat fleas, and Verjbitski (1908, p. 165) found *Ct. felis* or *canis* on 12% of rats (300) in Cronstadt. In Dar-es-Salam (German East Africa) *Echidnophaga gallinacea* accounted for 22% of fleas taken off rats (Schuberg and Manteufel, 1910). As may be seen from Table I, *P. irritans* has seldom been found except in San Francisco, where it was present to the extent of 9% of the total fleas taken.

*Distribution of rat fleas.*

The particular fleas found in greatest numbers upon rats seem to be determined by geographical position, including climate, and the habitat of the rats examined. The influence of species, whether *Mus decumanus* or *Mus rattus*, upon the parasite carried appears to be indirect and

brought about by a general difference in the mode of life and distribution of the two species.

In Table I below we have collected all the available quantitative data upon the subject which are based upon the examination of a sufficient number of fleas to permit the facts to speak for themselves. The results are, therefore, independent of the impressions of the individual observers. For the reason mentioned above (p. 123) such data are only forthcoming from comparatively few places, but they are sufficient to draw certain broad conclusions, as to the distribution of some of the commonest parasites infesting rats.

As an instance of the effect of geographical position independent of climatic conditions, the replacement of *Ceratophyllus fasciatus* by *Ceratophyllus anisus* in Japan (Kitasato, 1909) may be mentioned, and the occurrence of *Xenopsylla scopulifer* Rothschild only in Africa. The influence of temperature will be discussed when dealing with the distribution of *Xenopsylla cheopis*.

By habitat, we mean differences in conditions and mode of life depending upon whether the rats live in the fields, sewers, docks, barns, stores, granaries or poultry houses, &c. These varying surroundings will bring the rats into association with other animals and some amount of exchange of parasites may take place. Provided these parasites can flourish upon a diet of rat-blood, they may establish themselves locally on these rodents. An instance is the prevalence of *Ctenopsylla musculi*, the common mouse-flea, upon rats living under conditions in which rats and mice come together, such as in barns, provision stores and on some ships; the presence of fowl-fleas and dog-fleas in considerable numbers upon rats inhabiting poultry farms and stables is to be explained in the same manner. The large proportion, 52 %, of *Ctenophthalmus agyrtes* found by Martin and Rowland (1911) upon *Mus decumanus* living in the open in this country is another striking illustration of the case in point, the usual host of this flea being, according to Rothschild, the field vole.

(1) *Xenopsylla cheopis* (Rothschild). This flea was described by Rothschild (1903 and 1906) after examination of specimens taken from several small rodents in Egypt and the Soudan. It is now known to be the common rat flea in the tropical and sub-tropical parts of the world, occurring in some regions to the almost entire exclusion of other species. In India<sup>1</sup>, where rat fleas were very extensively examined in four places,

<sup>1</sup> The authorities for this and the following statements are given in the last column of Table I.

it formed nearly 100% of all the rat fleas found in three instances (Table I); in the fourth (Punjab) the same was true in the warmer months, 2% of *Ceratophyllus fasciatus* appeared in the cooler weather. In Manila, where, however, comparatively few fleas (42) were examined, all were identified as *Xenopsylla cheopis*.

In the warmer parts of the temperate regions, e.g. Mediterranean, Southern Japan, San Francisco and Australia as far south as Sydney, it occurs in varying proportion according to the time of year.

*Xenopsylla cheopis* is also stated to be common on rats in South Africa and Central and South America (Jordan and Rothschild, 1908).

One specimen has been found at Plymouth by Giles (1906) and this is the only recorded instance of its appearance in this country. We have no doubt, however, that it would be frequently found if, during September, a sufficiently extensive examination were made of rats from ships in English ports<sup>1</sup>.

The extensive census of rat fleas in various parts of India made throughout the year by the Plague Commission (1908, p. 266, 1910, pp. 460 and 524) shows a striking maximum for flea prevalence (*X. cheopis*) during some portion of the year, the mean number infesting rats increasing three to ten fold.

In those temperate climates (Japan, Sydney, Marseilles) where observations have been directed to the point, a seasonal prevalence of these fleas has also been observed, the numbers increasing greatly both actually and relatively towards the end of summer and declining with the onset of cold weather (Kitasato, 1909; Tidswell, 1910, p. 20; Gauthier and Raybaud, 1910, II.).

(2) *Ceratophyllus fasciatus* (Bosc.). This species is, according to Rothschild (1906, p. 483) and Tiraboschi (1907, p. 595), the common flea upon *Mus decumanus* and *Mus rutilus* in this country and in the northern and central parts of Europe. Galli Valerio (1900, I.), however, found it rarely in Lausanne. He believes that *Ctenopsylla musculi* is the usual flea on rats in this city and states that it is commonly present in Holland and at Halle and that he himself found it at Milan.

Recently Martin and Rowland (1911) found it to constitute about one half (48%) of 1057 fleas taken from *Mus decumanus* in Suffolk and Elstree (Herts.).

<sup>1</sup> Since this paper was written Dr Boycott has taken two specimens of *X. cheopis* off *Mus decumanus* captured at Guy's Hospital, London, S.E. The specimens were identified by Mr Rothschild.

TABLE I.

Locality	Source	Species	No. of rat fleas examined	Percentage of	
				Xenopsylla cheopis	Xenopsylla sootypluma
India: Bombay ..	Houses, gullies outbuildings	<i>M. r. ratus</i> and <i>M. decumanus</i>	216,000	100	—
Poona ..	Houses, out-buildings	<i>M. r. ratus</i>	240,000	100	4 only
Belgaum ..	Houses	„	380,600	100	—
Punjab...	Houses	„	54,000	98	—
Australia: Brisbane ...	—	<i>M. r. ratus</i> and <i>M. decumanus</i>	103	76·7	—
Sydney ...	—	„ „	100	81·0	—
„ ...	—	—	1926	61·8	—
San Francisco ...	—	<i>M. r. ratus</i> and <i>M. decumanus</i>	10,972	21·4	—
„	—	„ „	15,554†	32·1	—
„ (Oakland)...	—	<i>M. decumanus</i>	4140	23·2	—
Manila ...	—	<i>M. r. ratus</i> and <i>M. decumanus</i>	42	100	—
Africa: Dar-es-Salam ...	—	„ „	258	66·6	10·9
Japan: Yokohama, Tokio, and other large towns (winter)	—	—	2713	6·1	—
Yokohama ...	Ships	<i>M. r. ratus</i>	—	100	—
Awaji Island near Kobe (autumn)	—	—	835	45·5	—
„ „ (winter)	—	—	4617	20·2	—
Marseilles ...	Ships	<i>M. r. ratus</i>	250	25·6	—
„ ...	Town	<i>M. decumanus</i>	52	5·8	—
„ ...	Ships	<i>M. r. ratus</i>	2276	92·7	—
„ ...	Quays	<i>M. decumanus</i>	2424	33·0	—
„ ...	Town	„	4377	50·4	—
Tunis ...	—	<i>M. r. ratus</i> and <i>M. decumanus</i>	1242	83·6	—
Algeria: Algiers ...	—	„ „	2410	59·7	—
Constantine and Oran	—	„ „	428	35·9	—
Great Britain: Suffolk ...	Rats in the open	<i>M. decumanus</i>	584	—	—
„ ...	Rats' nests	„	215	—	—
Herts. ...	„	„	258	—	—

\* In some cases the fleas are not described by these authorities in accordance with the latest nomenclature; there is, however, seldom any doubt as to the species intended.

TABLE I (*continued*).

total fleas belonging to the various species\*

<i>Ceratophyllus fasciatus</i>	<i>Ctenophyllus muscicola</i>	<i>Ctenophyllus agrestis</i>	<i>Ctenophyllus felidis et ctenis</i>	<i>Echidnophaga gallinacea</i>	<i>Pulex irritans</i>	Observer
—	—	—	—	—	—	Commission for Investigation of Plague in India Report, 1907, p. 412; 1908, p. 297.
—	—	—	2 only	—	—	Ibid. 1910, p. 524.
—	—	—	—	—	—	Ibid. 1910, p. 461.
2 (winter only)	—	—	—	—	—	Ibid. 1907, p. 914.
—	17.5	—	5.8	—	—	Tidswell, 1903, p. 71.
10	8.0	—	1.0	—	—	„ „
10.9	26.7	—	0.5	—	0.05	„ 1910, p. 20.
68.1	4.5	—	0.5	—	5.6	Surgeon Rupert Blue, private communication, 1908.
52.0	4.6	—	1.8	—	9.4	Carroll Fox, 1910.
44.8	31.6	—	0.2	—	0.2	„ „
—	—	—	—	—	—	Herzog, 1905.
—	—	—	—	22.5	—	Schnberg and Manteufel, 1910.
66.5‡	27.4	—	0.3	—	—	Kitasato, 1909.
—	—	—	—	—	—	„ „
58.6‡	25.5	—	3.9	—	—	„ „
28.1‡	20.7	—	0.03	—	0.1	„ „
2.4	71.2	—	—	0.8	Gauthier and Raybaud, 1908.	
86.5	3.8	—	3.8	—	—	„ „ „
6.7	0.5	—	0.1	—	—	„ „ 1910.
51.5	10.2	—	5.2	—	—	„ „ „
35.1	7.9	—	6.4	—	—	„ „ „
5.1	10.1	—	0.4	—	0.1	Conseil, 1909.
13.5	20.1	—	6.3	—	0.4	Raynaud, 1909.
22.9	36.9	—	4.3	—	—	Billet, 1908.
55.5	—	44.4	—	—	—	Martin and Rowland, 1911.
47.0	—	53.0	—	—	—	„ „ „
33.3	—	66.7	—	—	—	„ „ „

† It is possible that some of these fleas may be included among those previously reported upon by Surgeon Blue.

‡ These numbers refer to *Ceratophyllus anisus*.

In warmer temperate regions *Ceratophyllus fasciatus* together with *Xenopsylla cheopis* forms the bulk of the rat-flea population, e.g. San Francisco, where out of a total of 10,972 rat fleas collected, 68 % were *Ceratophyllus fasciatus* and 21·4 % *Xenopsylla cheopis*. As a rule, in such climates, the proportion of the former increases relatively during the winter, and that of the latter during summer.

*Ceratophyllus fasciatus* has not been found in the warmer parts of India, but in the Punjab it appears during the cooler half of the year, November to March, and accounts for 2 % of the total.

In the Himalayas (Simla) this flea is common.

(3) *Ceratophyllus anisus* Rothschild. In Japan this flea appears to occupy the position assumed by *Ceratophyllus fasciatus* elsewhere (Kitasato, 1909), occurring to a relatively larger extent in the winter than in the summer, when *Xenopsylla cheopis* makes its appearance in some places in large proportion.

(4) *Ctenopsylla musculi* (Dugès). This is the flea which usually infests the house mouse, *Mus musculus*. Tiraboschi (1907, p. 60) found it on rats in all parts of Italy, but less frequently on *Mus decumanus* than on *Mus rutilus*. According to Verjbitski (1904) it is the flea most common upon rats in Cronstadt.

As may be seen from Table I, this flea furnishes a considerable contribution to the total fleas harboured by rats in many widely separated parts of the world: North America, Europe, Japan, Australia. It was rarely found in India.

It is sometimes very prevalent upon *Mus rutilus* from ships; on one occasion at Marseilles, among 250 fleas from this source Gauthier and Raybaud (1903) identified 178 as *Ctenopsylla musculi*.

(5) *Ctenophthalmus agyrtes* (Heller). According to Rothschild, this flea is common in England and Europe upon field mice and bank voles and occurs also on *Mus decumanus* when captured in the open. The recent observations of Martin and Rowland (1911) on the fleas from rats living on farms in Suffolk, Hertfordshire and Hampshire showed an unexpected proportion of this flea to be present (Table I). The rats examined were living in burrows in the fields or in corn stacks. It appears therefore that in these localities *Ctenophthalmus agyrtes* accounts for about half of the fleas infesting rats living under the conditions mentioned. Whether the same obtains in other rural parts of England is at present unknown.

Members of the above five species constitute the bulk of fleas, which up to the present have been shown to commonly occur upon rats.

One other flea, *Xenopsylla scopulifer* Rothschild, which, according to Schuberg and Manteufel (1910), replaces *Xenopsylla cheopis* to some extent in German East Africa, demands mention. It was described by Rothschild (1905) and obtained off *Saccostomus campestris* and *Mus auricomis* in Zululand. It is closely allied to *Xenopsylla cheopis*.

*The readiness with which various rat fleas attack man.*

1. *Xenopsylla cheopis*. That this flea readily feeds on man was observed by Tidswell (1903) and Gauthier and Raybaud (1903). This was confirmed by Liston (1904), who also collected many observations showing that, in the absence of its natural host the rat, *X. cheopis* is attracted to mankind. This fact was subsequently confirmed by the Commission for Investigation of Plague in India (*Report*, 1907, p. 472 and 1908, p. 249).

2. *Ceratophyllus fasciatus*. With regard to the readiness with which this flea attacks man considerable divergence of opinion has hitherto existed. According to Wagner (quoted by Tiraboschi 1904, p. 180), Tiraboschi (1904, p. 266) and Galli-Valerio (1907) this flea does not bite man. On the other hand, Gauthier and Raybaud (1903 and 1909) and McCoy and Mitzmain (1909) found that when hungry it fed on man with readiness.

We have made some hundreds of experiments on this question and are at a loss to understand the negative conclusion arrived at by Tiraboschi and Galli-Valerio.

The fleas used in our observations had been bred in the Institute and were kindly placed at our disposal by Professor E. A. Minchin. The fleas were caught in the breeding cages and placed separately in test tubes, which were kept in tins or jars in a dark cupboard covered with damp cloths to prevent undue drying of the atmosphere.

The fleas were starved for periods varying from 24 hours to 14 days before testing their inclination to feed on man. The tubes were inverted upon the naked skin of the arm of the subject and the flea carefully watched for a period of two minutes. If it did not bite during this time it was regarded as negative for our experiments. Frequently the flea attached itself instantly or within a few seconds and began to feed without delay; sometimes it took a little longer to get started, but usually if it were going to bite at all it did so before one minute had elapsed.

As soon as the flea attached itself the tube was removed and the behaviour of the insect was watched with a hand-lens. The position assumed was generally characteristic. After a preliminary investigation of the surface with the tip of the labium and the maxillary palps, the insect was seen to press its head firmly on to the skin and raise its abdomen steeply into the air. The mandibles and epipharynx, which constitute the "pricker" and enter the skin, are too small to be seen with a magnification of 10 to 20 diameters but the labium in which these parts are carried, can be clearly observed. As the "pricker" is pressed into the skin the slack of the bifurcated labium is either bent in the form of two bows or flexed as the fingers are in making a rest for a billiard cue. The insect is so much absorbed in the occupation of sucking, that it can be rotated 180 degrees around its pricker as axis without being detached. In a few cases in which, for some reason or other, notwithstanding the manifest efforts of the animal, no blood entered the stomach, this attachment of the insect was regarded as evidence of biting for statistical purposes. Usually, however, there was no difficulty in seeing the blood entering the stomach in rapid jets. A pink flush spreads over the anterior portion of the abdomen, which gradually reddens as the stomach becomes distended.

The fleas which did not bite usually wandered about during the two minutes they were under observation, and in some cases showed evident dislike, making efforts to crawl or jump back into the tube, away from the skin of the subject.

The results of 517 experiments with 364 fleas and 8 persons are given in Table II. The proportion of fleas which bit varied from 36% to 77%, giving a mean value for man of 59·6%. The variation between different people is large enough to warrant the conclusion that the suggested idiosyncrasy is a reality.

In order to obtain a satisfactory control for these experiments, a similar set were undertaken, using the rabbit and the rat as subjects; the results are also given in Table II. The experiments were in all details similar to the above, except for the expedients adopted for keeping the animal quiet during the observations.

The rabbit was rolled up in a cloth, and remained motionless on a table in a sleepy condition. The fleas were each allowed to remain for 2 minutes on the inside of the ear. 32 fleas were tried of which 23 bit, a proportion of 72%.

The rat was secured by a similar method. Two bandages were used, one for the neck and shoulders, and one for the thighs and legs, leaving a portion of the abdominal wall free. The area of skin upon which the

fleas were tried was shaved. The rat was laid on its back, the head comfortably supported with a pillow of cotton wool, and kept from rolling away by fastening the ends of the bandages to a board with drawing pins. The rat remained perfectly quiet and there was no difficulty in carrying out the observations. 101 fleas were tried, of which 59 bit.

Under the conditions of these experiments *Ceratophyllus fasciatus* fed upon man as readily as upon a rat.

TABLE II.

*Ceratophyllus fasciatus.*

Subject:-	C.J.M.	H.C.	A.M.	J.H.S.	H.Y.	G.F.P.	H.W.A.	S.R.	Summary	Rat	Rabbit	
Total No. of Exps.	161	118	39	33	52	51	40	23	517	101	32	
Positive...	...	106	65	30	12	36	25	19	15	308	59	23
Negative	...	55	53	9	21	16	26	21	8	209	42	9
% positive	...	65·8	55·1	76·9	36·4	69·2	49·0	47·5	65·2	59·6	58·4	71·9

*Cage experiments with Ceratophyllus fasciatus.*

The readiness with which *C. fasciatus*, if hungry, will attach itself to man was demonstrated in the following manner. The rat was removed from a flea breeding-cage, into which 100 fleas had been placed a few days previously. Four days later, the hand and arm of a number of persons were, at different times during the day, placed in the cage and allowed to remain there for two minutes. The results are given in Table III, where it is seen that in one case as many as 18 fleas jumped upon the arm, many of which could be felt at once to bite vigorously.

TABLE III.

*Cage experiment 16. xi. 10.*

Observer:-	C. J. M.	H.C.	A. M.	J. L. C.	H.Y.	G.F.P.	H.A.	H.B.	
No. of fleas found upon arm after 2 mins.	1 7	2 3	3 7	18	1 3	2 3	3	6 0	6 14

Further observations to test this point were made by putting the arm into cages in which the fleas were bred. These cages consisted of two compartments, separated from one another by coarse-meshed wire netting. The rat inhabited one compartment, and the other served as breeding ground for the fleas which could freely pass from one to the other. When the hand was placed in the compartment containing the

rat the result was negative, but when inserted into the other, although within six inches of the rat, numbers of fleas (in one case 40) hopped on to the arm, and most of them fed immediately.

The details of these experiments are set out in Table IV below.

TABLE IV.  
*Cage experiment 26. xi. 10.*

Observer	Cage No.	No. of fleas attaching themselves in 1 minute, outside rat compartment but within 6 inches of rat
C. J. M.	1	8
C. J. M.	2	40
G. K.	2	20

The bite of *Ceratophyllus fasciatus* was not, as a rule, followed by either irritation or swelling. In the case of one individual, however, the results were very distinct and lasted for several days. The photograph reproduced in Pl. II was taken 48 hours after 30 fleas of this species had been placed upon the arm. Every bite was followed by an inflamed papule surrounded by oedema. The irritation was considerable and lasted for some days.

3. *Ctenopsylla musculi*. According to Galli-Valerio (1900, *a* and *b*), Tidswell (1903) and Tiraboschi (1904, p. 282) this flea does not bite man. Verjbitski (1908, p. 164) came to the same conclusion. This observer made 40 experiments on himself, after starving the fleas for different periods, but in no case did they feed. On the other hand, McCoy and Mitzmain (1909), and Gauthier and Rayband (1910) found that this flea would occasionally bite man.

McCoy and Mitzmain's observations were made with 15 fleas. Each flea was tested on successive days for five minutes until it either died or escaped. With these opportunities eight of them were induced to feed on at least one occasion.

In our own experiments the fleas were bred in the Institute in cages with mice. From a cage started in December 1910, enough fleas were obtained on January 14, 1911, for a satisfactory experiment, viz.: 71 fleas. On the day following their removal from the cage only 46 were still alive. This species would appear to be less robust than either *Ceratophyllus fasciatus* or *Ctenophthalmus agyrtes*, a fact also noted by McCoy and Mitzmain.

With these 46 fleas, starved for 24 hours, the 111 experiments set forth in Table V were made; 4 of the fleas bit and remained attached

for several minutes, in fact until removed. In only one of these was blood found at the subsequent examination. 11 fleas which had given negative results were placed upon the shaved abdomen of a mouse; 9 of them fed. The same conditions obtained as in the case of *Ceratophyllus fasciatus* and the fleas were allowed to remain on the skin for two minutes only.

TABLE V.

*Experiments with Ctenopsylla musculi, 14. i. 11.*

Subject	H. C.	C. J. M.	S. R.	Summary	Mouse
No. of Experiments	36	47	28	111	11
Positive	...	1	1	2	9
Negative	...	35	46	26	2
% positive	...	—	—	—	3·6
					81·8

Our results are in agreement with those of McCoy and Mitzmain and Gauthier and Raybaud. This flea will occasionally bite man, but it evinces small inclination to do so.

4. *Ctenophthalmus agyrtes*. No observations to determine whether this species bites man have, as far as we know, been previously made.

The fleas for our experiments were obtained from rats and rats' nests examined during the investigation of plague among rats in Suffolk (November 1910 to January 1911) by Martin and Rowland (1911).

In all 98 experiments were made with 68 fleas and four persons (C. J. M., H. C., S. R. and A. M.); in every case the result was negative.

As control, some of the fleas, which would not feed upon man, were tested upon a rat. Unfortunately we omitted to do this until our supply was nearly exhausted and the number of fleas constituting the control was only 19. These 19 were placed upon the arm and allowed to remain for two minutes with negative result. Immediately afterwards they were placed upon the shaved skin of a rat (in the manner described above) under precisely similar circumstances as those in which they had failed to bite us. 11 fastened upon the rat and fed.

We have not yet succeeded in breeding a supply of *Ctenophthalmus agyrtes*, so that our experiments are not so numerous as with *Ceratophyllus fasciatus*, but they show that the former flea exhibits no inclination to feed upon man.

The following experiment described by Rowland and one of us (1911) illustrates the difference between *Ceratophyllus fasciatus* and *Ctenophthalmus agyrtes* as regards their appetite for man. A bottle containing 23 unidentified fleas, taken from wild rats three days previously,

was inverted upon the arm of one of the authors (S. R.). Eleven attached themselves and remained feeding; the remainder, 12, were shaken back into the bottle. The 11 which had fed were chloroformed and on examination were identified as *Ceratophyllus fasciatus*. The remaining 12 were placed upon C. J. M.; one fed and this also was identified as *Ceratophyllus fasciatus*. The 11, which had bitten neither observer, were found to consist of eight *Ctenophthalmus agyrtes* and three *Ceratophyllus fasciatus*.

#### SUMMARY.

(1) As far as is at present known, the great majority of the fleas infesting *Mus rattus* and *Mus decumanus* in different parts of the world, belong to either the species *Xenopsylla cheopis*, *Ceratophyllus fasciatus*, *Ceratophyllus anisus*, *Ctenopsylla musculi* or *Ctenophthalmus agyrtes* or are comprised of some admixture of these five species.

(2) *Xenopsylla cheopis* is the most prevalent in the tropics and sub-tropical regions and often occurs there to the almost complete exclusion of other species. It is common during summer and autumn in some of the warmer parts of the temperate zone, more especially in ports which have maritime intercourse with the tropics.

(3) In the cooler regions *Ceratophyllus fasciatus* is the most universally distributed flea and is associated with more or less of *Ctenopsylla musculi* and *Ctenophthalmus agyrtes* according to the locality and the habitat of the particular rats.

(4) In Japan *Ceratophyllus fasciatus* is replaced by *Ceratophyllus anisus*, a closely allied species.

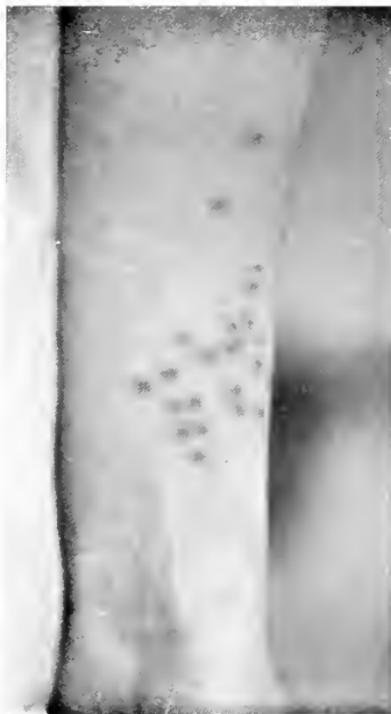
(5) The numerous other fleas which have been captured off rats are only occasional visitors.

(6) *Ceratophyllus fasciatus*, like *Xenopsylla cheopis*, readily bites man. Out of 517 experiments 308 fed, or 59% were positive. In 101 experiments, under identical circumstances with a rat, 59, or 58.4% of the fleas fed.

(7) The experiments with *Ceratophyllus fasciatus* were made upon eight persons and evidence was obtained of preference on the part of the insects for particular individuals.

(8) 111 experiments were made with 46 specimens of *Ctenopsylla musculi*; only 4 fed = 3.6%, whereas 9 out of 11 fed on a mouse.

(9) 68 specimens of *Ctenophthalmus agyrtes* were tried, in some cases upon three persons. None fed, whereas 11 out of 19 of the same fleas fed on a rat under identical conditions.



Forearm of A. M. 48 hours after 30 "Ceratophyllus fasciatus" had been placed upon it, 24 of the fleas having fed.



## GENERAL CONCLUSION.

*Xenopsylla cheopis* and *Ceratophyllus fasciatus* are the species of rat fleas which, when hungry, readily bite man. There is no reason to suppose that, other things being equal, *Ceratophyllus fasciatus* would not be as efficient an agent in the transmission of plague from rat to man as *Xenopsylla cheopis* has been shown to be in India.

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(29)

## A DIPHTHERIA-LIKE BACILLUS CAUSING CELLULITIS IN THE REGION OF A SPONTANEOUS FRACTURE IN A CASE OF TABES DORSALIS.

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### *Preliminary.*

THE interest of this case is due to the fact that the patient from whom this bacillus was isolated was a tabetic in whom a spontaneous fracture of his femur had occurred, and cellulitis developed at the level of the lesion due to a diphtheria-like bacillus. This organism was isolated and obtained in pure culture from the cellulitis and was found present in the films of the pus; also the patient's serum gave a complement fixation reaction with his own bacillus and for syphilis.

### *Clinical History.*

The patient was an adult man, suffering from tabes dorsalis and a fracture of the lower end of the left femur which occurred spontaneously as he was walking across the room. A sub-acute cellulitis developed at the level of the fracture and extended upwards towards the thigh. There was no external wound. The pus was obtained by puncture of the soft tissue with a sterile glass syriuge. The patient's leg was amputated, but he died shortly after the operation.

The only point of interest concerning the result of the autopsy is that the heart muscle and diaphragm, when stained with Scharlach R., were found to show diffuse fatty change.

Cultivations from the spleenic juice gave a negative result.

*Examination of the Pus.*

The pus obtained by puncture from the area of cellulitis was collected in a sterile tube. Film preparations showed pus cells and bacilli morphologically resembling the diphtheria bacillus in large numbers, chiefly extra-cellular, although many were intra-cellular. Cultivations on ox serum agar gave an almost pure growth of delicate semi-transparent colonies which proved to be a bacillus closely resembling the true diphtheria bacillus—morphologically. There were also a few colonies of a staphylococcus albus. The bacillus was found to grow well on solidified blood serum; the colonies were larger and more numerous than on serum agar. It grew badly on agar and any medium which did not contain blood serum. It stained well with Loeffler's methylene blue and was Gram positive. Young cultures on blood serum were tested with Neisser's stain and also films taken direct from the tissues, but with uniform negative results. Cultures of the true diphtheria bacillus were tested at the same time so as to control the results with Neisser's stain.

*Cultural Characters.*

The "sugar" reactions of the bacillus isolated from the patient's tissues were tested both before and after passage through guinea-pigs with similar results.

The bacillus grew in litmus milk with a slight production of acid after 48 hours, but no formation of clot and no increased production of acid at the end of ten days at 37° C. was noted.

It slightly, but distinctly acidified lactose, maltose and mannite without gas formation, but dextrose, cane sugar and raffinose were unaffected.

Graham-Smith, writing on this subject in the standard work on diphtheria<sup>1</sup>, quotes experiments to show that true diphtheria bacilli, whether virulent or non-virulent, invariably produce acid in the presence of glucose, generally with maltose and lactose, occasionally with cane sugar, but never with mannite.

The organism tended to die out unless frequently subcultured on a medium containing blood serum. The size of the colonies and general naked eye appearances of the cultures have not altered since the organism was originally isolated from the tissues. It grows slowly on gelatine at 22° C., without liquefaction of the medium.

<sup>1</sup> *The Bacteriology of Diphtheria*, edited by G. H. F. Nuttall and G. S. Graham-Smith, Cambridge University Press, 1908, p. 225.

*Blood Serum Reaction.*

The patient's serum gave a positive Wassermann reaction for syphilis and also a positive complement fixation reaction with his own bacillus.

The organism was taken up by normal leucocytes in the presence of normal serum very unequally, but with greater readiness in the presence of the patient's own serum.

*Pathogenicity.*

A young culture of this bacillus cultivated direct from the patient's tissues on ox serum agar (15% serum) when inoculated into guinea-pigs produced a fatal result within four days. There was local necrosis, but no lesions elsewhere in the body. The bacilli were seen in film preparations and were isolated from the tissues at the lesion and from the spleen.

The inoculation of the sub-cultures into two guinea-pigs produced death in these animals in 48 hours with similar post-mortem changes; a pure growth of the bacillus was obtained from the local lesion and from the spleen. The heart muscle of all the guinea-pigs was stained with Scharlach R. for fatty change, but with a negative result. This form of "degeneration" of the heart and diaphragm is now known to be the most common microscopical lesion met with in diphtheritic toxæmia and as I have shown elsewhere<sup>1</sup> occurs within sixteen hours of injecting the toxin.

Subsequent subcutaneous inoculation of guinea-pigs with this bacillus failed to produce a fatal effect, only a local abscess at the level of the lesion with ulceration of the tissues. Numerous diphtheria-like bacilli could be seen in films of the pus and were obtained in pure culture.

*The Action of a Prophylactic Dose of Diphtheritic Anti-Toxin  
on the Activity of the Bacillus.*

A guinea-pig, inoculated with a  $\frac{1}{8}$ th c.c. of a broth emulsion of a 24 hours' growth of the true diphtheria bacillus on blood serum, died

<sup>1</sup> "A Study of the Various Changes which occur in the Tissues in Acute Diphtheritic Toxæmia, more especially in regard to 'acute cardiac failure,'" *Brain*, Part CXIV, p. 227.

within 36 hours. At the autopsy, spreading oedema near the seat of inoculation was present together with a sloughing local lesion. Morphological diphtheria bacilli were seen in films from the pus, and a pure growth of the bacillus was obtained. The usual post-mortem phenomena—adrenal congestion, hydro-pericardium and bilateral hydro-thorax were present.

Two guinea-pigs received 500 units of diphtheritic anti-toxin and twenty-four hours later a similar dose of the *true* bacillary emulsion as employed in the case of the control guinea-pig, but showed no ill effects. In the guinea-pig which was inoculated with the test bacillus a local abscess developed at the seat of inoculation; similar abscesses were found in two guinea-pigs which previous to the inoculation with the test bacillus had received 500 units of diphtheritic anti-toxin. In each case the bacillus was isolated from the abscesses at the level of the lesion, but not elsewhere, and no fatal effect occurred.

#### *Production of Toxin.*

The bacillus was grown for one week at 36° C. in peptone broth free from glucose. The culture was then filtered under pressure through a Doulton candle, and when found to be sterile, 2 c.c. were injected subcutaneously into two guinea-pigs without any immediate effect being produced and the animals' tissues were free from disease when examined a week later.

#### *The Comparative Relationship of this Bacillus with the Bacilli met with in the Tissues of General Paralysis, Diphtheria, and certain other Infectious Conditions.*

The argument for and against the relationship of diphtheroid bacilli in the tissues of general paralytics to the disease itself does not concern us here, but the presence of a diphtheria-like bacillus in an acutely spreading suppurative lesion in a patient suffering from a disease so closely allied to general paralysis is of very great interest. This bacillus differs, however, from that described by Ford Robertson in that it does not stain with Neisser's stain, it fails to ferment dextrose and is markedly pathogenic for guinea-pigs<sup>1</sup>.

<sup>1</sup> Abstracted from the article by Dr Graham-Smith, *loc. cit.* p. 444.

*Diphtheria.*

The bacillus, when it was first isolated in the pus from the cellulitis, closely resembled the true diphtheria bacillus both morphologically and culturally.

It killed the first two guinea-pigs within four days, and the subcultures proved fatal to two guinea-pigs within 48 hours; since that time the bacillus has proved much less pathogenic, as referred to elsewhere.

There was, however, an absence of the common post-mortem phenomena associated with fatal diphtheria in guinea-pigs, which have been already referred to.

It failed to stain by Neisser's method, did not give rise to an extracellular toxin when growing in sugar free broth, and the action of the bacillus on the tissues of guinea-pigs was unaffected by diphtheritic anti-toxin. Other points could also be mentioned, but these are sufficiently important differential tests.

*Diphtheria-like Bacilli.*

Graham-Smith refers to various cases of suppuration produced by diphtheria and diphtheria-like bacilli, but I cannot find any reference to cellulitis, while even in the abscess cases there is no record of an organism similar to that under discussion.

In conclusion, this bacillus must be classed as an organism belonging to that large group of bacteria usually known as diphtheroid bacilli or diphtheria-like bacilli,—a group of organisms which every day appear to be assuming greater importance in the bacteriological world.

## ABSTRACTS OF OFFICIAL PUBLICATIONS, ETC.

*Many investigations relating to Hygiene and Public Health, possessing permanent scientific value, appear in Annual Reports of Medical Officers of Health, Blue Books and other Publications of Official Bodies within the British Empire. These are not easily available and as a rule no record of them appears in scientific journals or in an accessible form; consequently much valuable work is lost sight of. To obviate this difficulty the Editors of the "Journal of Hygiene" propose to publish from time to time abstracts of such publications, or parts of them, as appear of sufficient permanent interest. They will be glad to receive full authors' abstracts of Reports coming under the above description.*

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### ON THE BLEACHING OF FLOUR AND THE ADDITION OF SO-CALLED "IMPROVERS" TO FLOUR.

BY J. M. HAMILL, M.A., M.D. (CANTAB.), D.Sc. (LOND.).

*(Abstract of a Report to the Local Government Board, England.  
Food Reports No. 12.)*

#### 1.—BLEACHING PROCESSES.

THE practice of flour bleaching originated in this country about ten years ago; bleaching, however, is said to have been attempted in France two or three years prior to this. The practice was rapidly adopted by millers in the United Kingdom, in America, and elsewhere. Such bleaching is applied only to roller-milled flour. Numerous patents have been granted for bleaching appliances and for the application of various chemicals to the bleaching of flour. Ozone, chlorine, oxides of chlorine, bromine, nitrogen peroxide and other oxidising substances have been suggested and tried as bleaching agents. Of these substances nitrogen peroxide is the only one which has given satisfactory results, and all commercial bleaching of flour at the present time depends on the use of this substance. It can be produced in various ways and from a variety of materials. Two methods of production, which may conveniently be termed the chemical and electrical methods, are in use commercially at the present time. In the first of these nitrogen peroxide is produced by the action of nitric acid on ferrous sulphate, the amount of gas formed being controlled by regulating the supply

of acid and ferrous sulphate. A current of air charged with the gas is led through a revolving reel or "agitator," as it is called, through which a stream of flour is continually flowing. The flour in its passage is thoroughly exposed to the action of the nitrogen peroxide, and is bleached when it emerges from the agitator. In actual practice this process possesses certain commercial disadvantages. The apparatus requires constant attention if the degree of bleaching is to be properly controlled; overbleaching and condensation of acid resulting in local staining of the flour are liable to occur, while in the hands of ordinary workmen the use of strong acids is not entirely free from risk. As regards expense, it compares unfavourably with other methods, some of which are said to be worked at one-tenth of the cost of the acid process.

Another chemical process depends on the production of nitrogen peroxide from the combustion of ammonia in air. A current of air, mixed with ammonia gas obtained from an iron cylinder containing liquid ammonia, is pumped through a narrow platinum tube. The tube becomes red hot, and the oxygen of the air and the ammonia interact with the production of nitrogen peroxide. The air charged with nitrogen peroxide is passed together with flour through an agitator in the way already described. The apparatus is compact, and the process is free from the drawbacks attendant on the use of nitric acid and ferrous sulphate. It is suitable for mills where the output of flour is small. Nitrosyl chloride has also been proposed as a source of nitrogen peroxide.

The electrical methods of bleaching depend upon the fact that an electrical sparking discharge in air results in the combination of a certain amount of the nitrogen and oxygen of the air to form nitrogen peroxide. Electrical methods of bleaching are rapidly superseding chemical processes on account of their simplicity in operation and the greater accuracy with which the degree of bleaching can be controlled and overbleaching avoided. Condensation of acid, resulting in local staining of the flour, is said to be less likely to occur than in the chemical processes. Condensation is said to be prevented if the room in which the agitators are placed is kept warm.

In one form of electrical apparatus two metallic electrodes connected with a suitable source of current, generally a small dynamo, are alternately brought into contact and separated, an intermittent flame-discharge occurring at each separation of the electrodes. Air is pumped over the electrodes, and at each flash becomes charged with nitrogen

peroxide. The concentration of nitrogen peroxide in the air can be regulated, within limits, by altering the amount of air pumped over the electrodes. In another form of apparatus, electricity supplied from a dynamo is transformed to a potential of 10,000 volts or more and conveyed by well-insulated conductors to fixed metallic electrodes (generally four pairs) between which a continuous sparking discharge occurs. In another part of the apparatus provision is made for the production of a brush discharge, and a current of air is pumped first over this and then over the sparking electrodes. In its passage through the brush discharge, part of the oxygen of the air is ozonised, and in its passage over the electrodes nitrogen peroxide is produced. The air, charged with nitrogen peroxide and ozone, is brought into contact with flour in an agitator in the usual way. It is now generally believed that ozone has no bleaching action on flour, and although originally an electrical bleaching machine was constructed with a view to the production of ozone, in the belief that this gas was the active bleaching agent, any bleaching which occurred was probably due to nitrogen peroxide formed simultaneously with the ozone. It may here be noted that the introduction of electrical methods of bleaching has been accompanied by extensive litigation, dealing with patents and patent rights<sup>1</sup>.

In these electrical bleaching machines the degree of bleaching obtainable can be varied either by altering the amount of air passing through the apparatus or by varying the strength and frequency of the electrical discharges. Quite recently a form of apparatus has been introduced which, it is claimed, enables the bleaching process to be controlled with greater precision than in any of the foregoing processes. In this apparatus a current of air under pressure is blown from a fine jet through an electric arc formed between two brass electrodes. A flame is thus produced the size of which, and, consequently, the amount of nitrogen peroxide generated, can be regulated with some accuracy by varying the strength of the current supplied to the machine<sup>2</sup>.

Millers are guided entirely by the appearance of the issuing flour in regulating and controlling the bleaching apparatus; they have no

<sup>1</sup> Flour Oxidising Company, Ltd. v. J. & R. Hutchinson (*Times*, 28th April, 1909).

<sup>2</sup> Commonly a current of 7 to 10 ampères at 100 volts is transformed to about 14,000 volts, at which potential the discharge occurs. Two to five cubic feet of air per minute, according to the pressure (generally about five inches of mercury), pass through the apparatus, which is capable of bleaching two to three tons of flour per hour. The inventor was, however, unable to give me any information as to the amount of nitrogen peroxide which the apparatus contributed to the air.

knowledge of the actual amount of nitrogen peroxide produced or used. Examination of the bleaching gas at different mills was made in course of my inquiry, and it was found that very different degrees of concentration of nitrogen peroxide were employed.

## 2.—CONSIDERATIONS INFLUENCING THE AMOUNT AND DEGREE OF BLEACHING.

The practice of millers as to the bleaching of flour varies with the circumstances of their trade. In Ireland, a demand exists for a very white flour; a taste for such flour probably arose on account of the importation of American flour, which "ages" naturally during transit, and in this way becomes extremely white. For scone-making and for household purposes generally, a white flour is also desired; it is stated that the yellow tinge which baking powders containing soda in excess may give to scones is rendered less apparent by the use of a very white flour. Millers, therefore, who supply places where for these or other reasons there is a particular and constant demand for very white flour, naturally find bleaching of special service. Apart from this, it has to be remembered that it may be cheaper for the miller to utilise wheats which normally would yield a comparatively dark flour. The number of such wheats now available for the miller is large, and some of them yield flour which possesses excellent baking qualities but is inferior in consequence of the importance which has come to be attached to colour. Among the wheats which yield flour for which on this account bleaching is considered specially useful, are certain Indian wheats, Hard Plate, Walla Walla, durum, and some varieties of Russian wheat. But even the naturally whitest flours may be bleached for districts where there is a demand for excessively white flour.

There are also other ways, to be referred to later, in which bleaching may enhance the value of particular flours to the miller. In these circumstances considerable differences in the practice of bleaching obtain not only in mills in different parts of the country, but frequently, also, in the same mill from day to day.

It must not be supposed that bleaching in this country has yet become universal, even amongst the larger firms. Many millers have never bleached flour for sale. Some of these are at present only deterred from installing bleaching plants by considerations of expense. Others have had bleaching plants installed but have discontinued using them—in some cases for the reason that they were unwilling to pay

the patentees the sum demanded for permission to use the process. Other millers who have abandoned bleaching have reconsidered their decision, and have resumed the practice. Bleaching has been carried on uninterruptedly since its adoption by certain millers, who have found it to their advantage commercially.

It appears to be the custom of many millers, when using a bleaching plant, to bleach all the flour produced. This, of course, would necessarily be the case whenever a straight run<sup>1</sup> was being milled and bleached. Some millers state that although they bleach their whole output they make a distinction between the lower and higher grades<sup>2</sup>, bleaching the latter more than the former. They believe that the best results from bleaching are obtained with the higher grade flours, and that the bleaching of lower grades, unless carefully done, is liable to mar rather than improve the appearance of the flour. In some mills, where a blend containing, say, Indian wheat is used, a larger proportion of flour from Indian wheat may appear at the bottom ("tail") of the mill, *i.e.* in the lower grades, than at the top of the mill. In this case the lower grades may be subjected to stronger bleaching than the higher grades. In certain cases the miller has found that his customers prefer high grade flour unbleached, and has, in consequence, restricted bleaching to low grades only.

The degree to which the bleaching of flour is carried depends on the kinds of wheat used; on whether the demand to be met is for very white flour or for flour with a slight yellow tinge or "bloom," and on similar considerations. Many millers content themselves with lightening the colour till only a slightly yellow tinge is perceptible—merely "touching" the flour, as they term it. Others are not satisfied with this but bleach until the flour is quite white. Certain millers consider this to be excessive, and assert that the dead whiteness thus obtained is unnatural and unpleasant.

Some millers, *e.g.*, in Glasgow, where baking and milling methods differ somewhat from those in other parts of the country, do not bleach flour from spring wheat but bleach winter wheat flours, although in general these are whiter than those obtained from spring wheat. These firms largely supply bakers who are accustomed to the tinge or

<sup>1</sup> By the term "straight run" is meant the whole of the flour produced in the mill without any separation into different grades.

<sup>2</sup> Here and elsewhere when the text requires it, such terms as "high," "low," "good" or "poor" when applied to grade or quality are used in the trade sense, and not in reference to food values.

"bloom" natural to spring wheat flour, and have come to regard it as characteristic of this class of flour. On the other hand, these bakers prefer winter wheat flour, which is used in the "doughing" stage of baking, to be as white as possible. Winter wheat flour is also used for home baking and scone making, and for these purposes is desired specially white, consequently it is the common practice of millers possessing bleaching plants, and milling spring and winter wheats separately, to bleach only the winter wheat flours as white as possible, and to leave the spring wheat flours untouched.

### 3.—THE EFFECT OF BLEACHING ON THE APPEARANCE AND OTHER PROPERTIES OF FLOUR.

The precise effect upon flour of bleaching by nitrogen peroxide is a matter of considerable dispute amongst millers, bakers and others who have given attention to this question, and who either favour or oppose the practice. The most obvious effect of nitrogen peroxide is the change of colour which it produces in the flour, but other more subtle changes, such as alterations in the baking qualities of the flour, are also attributed to its use.

The chief opponents of bleaching in the trade are many bakers, flour importers, and those millers who for one reason or another do not bleach. Bakers are not accustomed to combined action in matters of this kind, but in Glasgow, in 1906, the principal bakers issued a circular protesting against the bleaching of flour.

*The Effect of Bleaching on Baking Qualities.*—A number of investigations have been made on this point, especially in America. In many cases no differences have been observed in respect of the baking qualities between bleached and unbleached flour; in cases where differences have been observed they are small, and may well be within the limits of experimental error, or be due, where the baking qualities appear to be improved, to the slight drying of the flour during the bleaching process, or, where the baking qualities appear to have deteriorated, to excessive bleaching.

Avery<sup>(3)</sup> was unable to detect any difference in the baking qualities of bleached and unbleached flour, and Alway and Pinckney<sup>(2)</sup> have confirmed his observations.

Snyder<sup>(15)</sup> came to the conclusion that bleaching has practically no effect on the baking qualities of a flour. In some of his experiments slightly larger loaves were obtained from bleached flour than from

unbleached flour; this he attributed to slight drying of the flour during bleaching.

Ladd and Stallings<sup>(10)</sup>, on the other hand, assert that the gluten of flour deteriorates as a result of bleaching, and the water-absorbing capacity of the flour diminishes, consequently a larger loaf can be produced from unbleached than from bleached flour.

Amongst the bakers and millers (both bleaching and non-bleaching) whom I have visited the opinion is common that ordinary commercial bleaching produces little or no effect on the baking qualities of flour.

*Conclusions as to the effect of bleaching upon flour.*—It seems probable that bleaching, when carefully carried out, produces no appreciable effect upon the baking qualities of flour.

It may be true that by mere bleaching it is impossible to make the lowest grades of flour equal in appearance to the highest grades, but there can be no doubt that bleaching is capable of improving the colour and therefore the commercial value of the lower grades of flour. This is borne out by the common practice of bleaching the whole output of the mill, both high and low grades; also by statements of manufacturers of bleaching apparatus that bleaching improves both high and low grade flours, and enables the miller to produce "a longer length of patents," and further by the fact that some millers bleach only their lower grades of flour. Bleaching would seem to improve, from the miller's point of view, the colour of the whole output of the mill, although the improvement may be less marked in the lower than in the higher grades which are practically free from offal.

That this improvement represents a pecuniary gain to the miller is evident from the longer patents—*i.e.*, the larger percentage of high grade flour—which can apparently be obtained as a result of bleaching. This advantage must be remembered in connection with the assertion that the price obtainable per sack is not increased by the bleaching process.

It is probable that bleaching is of assistance in maintaining uniformity in the appearance of flour milled from different wheats, but at the same time it enables the miller to use cheaper wheats and to transform what would otherwise be a dark-coloured cheap flour into a whiter, higher-priced article, the gain due to the enhanced price going to the miller. It is said that a saving of 6d. to 1s. per sack may thus be effected by the miller.

Bakers, importers and others judge flour largely on its appearance and are accustomed to associate whiteness with high commercial quality

and with care and attention in the choice and milling of the wheat. Owing to bleaching, colour is no longer a trustworthy criterion, and a purchaser may be supplied with an article of a different quality from that which he believes he is receiving. Millers are, of course, aware of this, and show a disinclination to inform their customers that their flour is bleached.

There seems no sufficient evidence that mere bleaching can counteract defects in flour due to unsoundness in the wheat from which it was milled.

#### 4.—CHEMICAL CHANGES OCCURRING IN FLOUR AS A RESULT OF BLEACHING BY NITROGEN PEROXIDE.

Although very little is known with certainty regarding the chemical changes which occur in flour on treatment with nitrogen peroxide, I may here refer to results which have been obtained, particularly in America, by numerous observers whose investigations have been published.

It has been ascertained that a watery extract of bleached flour reacts to the Griess-Ilosvay test, showing the presence of a nitrite-reacting substance as a result of treating the flour with nitrogen peroxide. This reaction is not given by ordinary unbleached flour when care is taken to prevent its contamination with nitrates from the air or other sources. Warington<sup>(17)</sup> drew attention to the delicacy of this test as a means of detecting the presence of nitrates, and, later, Willard and Ladd employed it in connection with the examination of flour. Alway and Gortner<sup>(18)</sup> have described a method of applying this test to flour, and are convinced of its reliability, if the precautions enumerated by them are observed. Weil<sup>(19)</sup>, however, has recently stated that certain flours which have not been bleached respond to the Griess-Ilosvay test, and recommends instead that the flour be exposed to the action of sulphuretted hydrogen for one hour. Unbleached flour is unaltered by this treatment, whereas bleached flour becomes darker and acquires the colour it possessed before bleaching. Shaw<sup>(20)</sup> has proposed an alternative test, which has as its object the detection of nitro-derivatives in the flour. A concentrated alcoholic extract of bleached flour, treated with a drop of a solution of diphenylamine in sulphuric acid, gives a blue colouration. Alway and Gortner<sup>(18)</sup> have not found this test reliable in their hands.

The amount of nitrite-reacting substance, which can be demonstrated in bleached flour, varies considerably in different samples. In some

cases only traces can be detected; in others as much as 40 parts of nitrite (expressed as sodium nitrite) per million of flour have been found. The results obtained are variously given in parts of nitrite (expressed as sodium nitrite) or in parts of nitrite-nitrogen per million or per ten-million parts of flour.

Nitrates are also formed as a result of bleaching. When bleached flour is made into bread, from one-half to two-thirds of the nitrite present in the bleached flour disappears, and an increase in nitrates is said to occur. In biscuits practically all the nitrite disappears on baking. Alway and Pinckney<sup>(2)</sup> have found that no relation exists between the amount of nitrite which may remain in bread and that originally present in the flour; sometimes no nitrite may be left in the bread. Snyder<sup>(15)</sup> has suggested that the nitrite-reacting material in flour is either ammonium nitrite or a volatile compound of similar nature.

From the work of Fleurent<sup>(6)</sup> and others the bleaching of flour appears to be due to the destruction of yellow colouring matter dissolved in a thin layer of oil which surrounds each granule of starch. Ladd<sup>(9)</sup> has stated that changes in the appearance and in the composition of the oil, as evidenced by a lowered iodine number and by the introduction of nitrogen into the oil, result from the process of bleaching by nitrogen peroxide. The oil of flour, which had aged naturally for nine months, preserved its iodine number unaltered, whilst the iodine number of oil from the same flour, bleached with nitrogen peroxide and aged for the same length of time, had diminished considerably. Fleurent<sup>(6)</sup> also found that bleaching decreased the iodine number of flour oil, and states that the whiteness obtained by the action of the nitrogen peroxide on flour is due to the white starch granules becoming more obvious as a result of the decolourisation and increase in transparency of the oil film surrounding them, whereas the whiteness resulting from age is due to conversion of the oil into fixed fatty acids which are white in themselves.

Gill<sup>(16)</sup> asserts that bleaching in addition to lowering the iodine number converts a portion of the olein into elaidin.

Gustav Mann<sup>(16)</sup> states that the oil of flour is altered by treatment with nitrogen peroxide in such a way as to make it more difficult to stain with scarlet R or Soudan 3 than oil from unbleached flour.

The acidity of flour is stated by Balland<sup>(1)</sup> to increase as a result of treatment with nitrogen peroxide, but Alway and Pinckney<sup>(2)</sup> state that this is not the case if less than 50 c.c. of nitrogen peroxide per kilogram of flour be used.

It has been suggested that the formation of diazo- and nitro-compounds may result from the interaction of nitrogen peroxide and the gluten or protein of the flour. Skraup<sup>(4)</sup> and others have shown that nitrous acid can attack and profoundly alter the composition of proteins. Gustav Mann<sup>(6)</sup> states that the amount of nitrite present is no indication of the extent to which the protein may have been attacked, and draws attention to the possibility of the destruction of important amino-groups in the protein molecule as a result of bleaching, whereby its value as a foodstuff would be greatly diminished. Folin<sup>(6)</sup> concurs with this view, and believes that nitro-compounds may be formed as a result of the action of nitrogen peroxide on the protein of flour.

Other investigators maintain that little if any chemical change in the flour, other than the decolourisation of the oil and the presence of traces of nitrite-reacting material, can be detected as a result of treatment with nitrogen peroxide. Avery<sup>(3)</sup> has also come to this conclusion, and is of opinion that only in overbleached flour can appreciable changes, such as the diminution of the iodine number, be detected. Snyder<sup>(5)</sup> has likewise failed to obtain any change in the iodine number as a result of bleaching, and considers that Ladd is not justified in his conclusion that nitrogen is introduced into the flour oil by bleaching, since the oil from wheat flour normally contains a small amount of nitrogenous matter which is not increased by bleaching. He further maintains that in commercial bleaching only sufficient nitrogen peroxide is used to bleach the flour, and that the risk of forming diazo- and nitro-compounds is negligible. He also asserts that practically all the nitrogen peroxide brought into contact with the flour in the commercial process of bleaching can be accounted for, either in the air leaving the agitator or as physically combined (adsorbed) in the flour, and he attributes bleaching to the catalytic action of the nitrogen peroxide, which does not itself chemically combine with any of the constituents of the flour. Wesener and Teller<sup>(9)</sup> are of the opinion that no change except as regards colour is produced in flour as a result of commercial bleaching. They admit, however, that changes in the composition of the protein and fat may be caused by overbleaching.

The amount of nitrogen peroxide required to produce bleaching has also received attention. Avery<sup>(3)</sup> states that the nitrogen peroxide produced by 3 c.c. of nitric oxide in 3 litres of air will bleach 1 kilogram of flour; maximum bleaching was attained with 40 c.c. of nitric oxide; with amounts above this the colour of the flour deteriorated. Hale<sup>(7)</sup>

considers that the best results are attained by using 30 c.c. of nitric oxide to 1 kilogram of flour. Fleurent<sup>(6)</sup> states that according to the kind of flour from 15 to 40 c.c. of nitrogen peroxide is sufficient for 1 kilogram of flour. Snyder<sup>(15)</sup> found that 5 c.c. of nitrogen peroxide was sufficient to bleach 1 kilogram of flour; and Alway and Gortner<sup>(16)</sup> state that from 100 to 150 c.c. of nitric oxide are required to produce a maximum bleaching effect.

Shepard<sup>(16)</sup> points out that millers do not know how much nitrogen peroxide they use, but judge entirely by the colour of the flour, and that, relatively, large amounts (more than 100 parts of nitrogen peroxide per million of flour) may be absorbed and the flour remain a strictly commercial article not overbleached. He considers that in ordinary commercial bleaching 36 to 72 parts of nitrogen peroxide are absorbed by a million parts of flour and that even larger quantities may be required in the case of highly coloured flours.

Various observers have found very diverse amounts of nitrates in flour as a result of laboratory treatment with different amounts of nitrogen peroxide. A few of the values obtained are given in the table below. The figures in brackets represent the amount of nitrogen peroxide in cubic centimetres added to one kilogram of flour; the other figures opposite these represent, approximately, the parts of nitrite (expressed as  $\text{NaNO}_2$ ) found in a million parts of flour after treatment with the respective volumes of nitrogen peroxide:—

Alway and Gortner <sup>(16)</sup> .	Hale <sup>(7)</sup> .	Snyder <sup>(15)</sup> .
(5 c.c.) 6 parts.	(10 c.c.) 7 parts.	(5 c.c.) 2 parts.
(25 c.c.) 20 parts.	(20 c.c.) 8 parts.	(25 c.c.) 5 parts.
(50 c.c.) 30 parts.	(50 c.c.) 36 parts.	(50 c.c.) 16 parts.
(100 c.c.) 40-60 parts.	(100 c.c.) 40 parts.	(100 c.c.) 20 parts.

The results of laboratory experiments on the same point will be found in Dr Monier-Williams' report (see *This Journal*, xi. p. 167).

The amount of nitrite-reacting material in bleached flour tends to diminish with lapse of time when a certain degree of bleaching has been exceeded.

Snyder<sup>(15)</sup> found in one sample, bleached with 250 c.c. of nitric oxide per kilogram of flour, that 24 days after bleaching the amount of nitrite had decreased by one-half, i.e., from 8 parts per million to 4 parts per million; the latter amount can be obtained by 25 c.c. of nitric oxide. Alway and Gortner<sup>(16)</sup> observed that nitrates in bleached flour rapidly diminish in amount when the flour is kept if more than 50 c.c. of nitric

oxide per kilogram of flour has been used for bleaching. When less than this amount of nitric oxide has been used, no appreciable change in the nitrite content of the flour takes place on keeping.

The amount of nitrite-reacting material present in flour, as indicated by the Griess-Ilosvay test, is not necessarily any criterion of the degree of bleaching or of the amount of nitrogen peroxide which may have been absorbed by the flour. It would appear from experiments by Snyder<sup>(15)</sup> and by Alway and Gortner<sup>(16)</sup> that the nitrite content of flour varies with the degree of bleaching only up to a certain point, and beyond this is not permanently increased by further bleaching. The latter investigators have observed an approximate proportionality between the amount of nitric oxide used for the production of nitrogen peroxide and the nitrite produced up to 50 c.c. of nitric oxide per kilogram of flour. Thus, 2 c.c. of nitric oxide produced about 3 to 4 parts of nitrite per million of flour; 5 c.c. about 6 parts per million, 25 c.c. about 20 parts per million, and 50 c.c. about 30 parts per million. Larger amounts than this produce an increase in the nitrite content which is only temporary, and, as already mentioned, rapidly diminishes on keeping. It is, therefore, not justifiable from a mere determination of the nitrites in flour to draw any conclusions as to whether the flour has been subjected to excessive bleaching.

The work done on the chemical changes in flour as a result of bleaching has been incomplete, and the conclusions of the different investigators contradictory in several respects, it was arranged that some of the more important considerations should be investigated experimentally by Dr Monier-Williams (see *This Journal*, xi. p. 167). The results which he has obtained should be studied in connection with the above review. It would appear from his experiments that maximum bleaching is attained with 30 to 100 c.c. of nitrogen peroxide per kilogram of flour. In freshly bleached flour the amount of nitrite is proportional to the nitrogen peroxide used, amounting to about 30 to 40 per cent. of it. In the more highly bleached samples a decrease in nitrite was observed after a lapse of some days, but, practically, no alteration in the nitrite content of slightly bleached samples occurs. It appears that about 60 per cent. of the nitrogen absorbed as nitrogen peroxide can be extracted by water in the form of nitrites and nitrates; the remainder has probably entered into combination with or is absorbed by various elements of the flour. Dr Monier-Williams' work shows clearly that, with higher degrees of bleaching, definite changes are produced in the constitution of the flour. Among these are an

increase in soluble proteins and carbohydrates; the oil of the flour acquires the character of an oxidised oil, and about 6 to 7 per cent. of the nitrogen introduced into the flour as nitrogen peroxide is absorbed by the oil. No evidence of the formation of diazo-compounds was forthcoming.

*The Value of Chemical Methods in the Detection of Bleached Flour.*—The chemical change most easily demonstrated in flour as a result of bleaching is the formation of nitrites. The Griess-Ilosvay reaction when properly carried out affords an exceedingly delicate test for the presence of nitrites, and on this account has been largely used for the detection of nitrites in flour. Flour which gives a positive reaction with the Griess-Ilosvay test is commonly assumed to be bleached, but to infer bleaching from the mere occurrence of the nitrite reaction is in many cases not permissible.

In the first place, owing to the great delicacy of the test, care has to be taken that the materials used for the test, especially the water used for extracting the flour, are perfectly free from all traces of nitrites. Distilled water commonly contains nitrites, but in the majority of cases, tap water is practically free from nitrites, and when boiled over an electric heater to free it from dissolved oxygen, which may affect the test adversely, is suitable for use in the estimation of nitrites. The test itself should be performed in a room in which no flame is burning, since the air may in this way become contaminated with nitrous acid.

Flour readily absorbs nitrous acid from the air, and in manufacturing towns amounts easily appreciable by the Griess-Ilosvay test may gain access to the flour. In Bulletin 206 of the Laboratory of the Inland Revenue Department, Canada, reference is made to a sample of flour originally free from nitrites which was exposed to the air on the laboratory roof; after ten days it was found to contain 1·6 parts per million of nitrite and after 25 days 3 parts per million. In this case, however, the four ventilating flues from the laboratory in which all kinds of work were being done, opened on to the roof, so that at times the air over the roof probably contained large amounts of nitrous acid.

In the course of my inquiry samples of flour milled by firms who possess no bleaching plants have been found to contain as much as 0·5 part per million of nitrites (expressed as  $\text{NaNO}_2$ ). On the other hand samples known to have passed through a bleaching apparatus have been obtained which gave amounts of nitrites lower than 0·5 part per million.

For practical purposes, however, it may be presumed with some probability that when more than one part of nitrites per million is present the flour has been bleached; with quantities much above this the inference of bleaching amounts to practical certainty. On the other hand, when flour has been submitted to the Griess-Ilosvay test and has been found free from nitrites, there can be little doubt as to its being unbleached.

*Nitrites in Flour Sold in this Country.*—As a result of examinations of samples of flour taken under the Sale of Food and Drugs Acts, 12 samples of flour purchased in shops in Glasgow were recently reported by the Public Analyst for the County of Lanark to contain nitrites in varying amounts estimated at from 0·01 to 1·2 parts per million.

The Public Analyst for the County of Nottingham recently examined a number of samples of flour and found them to contain an average of six parts per million of nitrogen peroxide. The Public Analyst for the County of Chester examined, in 1909, 22 samples of flour of which 14 were bleached. The Public Analyst for Kingston-on-Hull, as a result of the examination of 20 samples of flour, found that 11 contained nitrites.

Information has kindly been supplied to me by medical officers of health and several other public analysts of the result of examination for nitrites, by the Griess-Ilosvay test, of flours purchased at retail shops or sold to bakers in different parts of England. Out of 157 samples 40 were reported to be free from nitrites and 117 contained nitrites. Of these 117 samples—

72 contained more than 0·5 parts of  $\text{NaNO}_2$  per million of flour.

35	"	"	1·0	"	"	"
4	"	"	2·0	"	"	"
2	"	"	3·0	"	"	"

None contained more than 4 parts per million.

Samples of flour were also taken by me at different mills immediately after being bleached and their nitrite content estimated. Out of 13 such samples only one contained as much as 4 parts per million of nitrites (expressed as  $\text{NaNO}_2$ ), one contained 3·5 parts per million, one contained 2 parts per million, four contained 1·5 per million, and the remaining six samples 1 part, or less, of nitrites per million. The amount of nitrite present in these samples is approximately of the same order of magnitude as that found in the nitrite-containing samples obtained in the ordinary way in retail shops and of bakers throughout the country.

These results would appear to show that, at the present time, flour bleached in this country contains somewhat less nitrite than is given in recorded results of analyses of flour bleached in the United States and Canada.

Alway and Gortner<sup>10</sup>) examined a number of samples of bleached flour in the United States and found the average amount of nitrites present in the samples to be 6·3 parts per million. Hale<sup>11</sup>) also asserts that in the United States the average amount of nitrite-nitrogen in bleached flour is about one part per million, *i.e.*, about 5 parts of nitrite (as sodium nitrite) per million of flour, and Wesener and Teller<sup>12</sup>) consider this to be the average nitrite content of bleached flour. In Bulletin 206 of the Laboratory of the Inland Revenue Department, Canada, a series of analyses of flour is given in which amounts of nitrites as high as nine parts per million have been detected. Several samples contained amounts above four parts per million. Out of 223 samples, however, 148 gave no reaction for nitrites.

*Nitrites in Imported Flour.*—A number of consignments of flour have recently been sampled on arrival in this country from abroad (chiefly the United States and Canada), and have been examined by Dr Monier-Williams for the presence of nitrites. Out of 70 such samples 13 were found to be free from nitrites and 57 contained nitrites. Of these 57 samples—

34	contained more than 0·5 parts of NaNO <sub>2</sub> per million of flour.				
24	"	"	1·0	"	"
11	"	"	2·0	"	"
4	"	"	3·0	"	"
2	"	"	4·0	"	"

None contained more than 4·5 parts per million. The amount of nitrite present in these samples is approximately of the same order of magnitude as that found in samples bleached in this country.

##### 5.—THE ADDITION OF OTHER SUBSTANCES TO FLOUR. FLOUR "IMPROVERS," SO-CALLED.

As my inquiry progressed it became clear that, besides the question of bleaching, other additions to flour had to be considered, in particular the following:—

*Water.*—In some cases water may be added to flour in the form of a very fine spray by means of an "atomiser," or other suitable apparatus. The spraying may take place upon the finished flour or at various

stages during the milling process; usually the latter, since more intimate incorporation is thereby ensured. This treatment is stated to improve the baking qualities of wheat in certain cases and to facilitate the milling processes. Incidentally it is possible in this way to add about one or two per cent. of moisture to wheat already containing 15 per cent. of water. This represents a considerable gain to the miller. I am informed that in some cases glycerine may be added to the water to assist the flour to retain the added moisture.

*Phosphates and other "Improvers."*—The work of Wood and others<sup>(21)(22)</sup> indicates that the properties of gluten can be modified by the addition of certain salts and acids to flour. Wood also states that the ash of strong wheats is richer in phosphates than that of weak wheats. Endeavours are being made at the present time to improve the baking qualities of certain flours by treating them with various salts and acids with the object of improving the quality of the gluten so as to increase the strength and water-absorbing capacity of the flour. One of the results of this is that the number of loaves which it is possible to obtain from a sack of flour is increased. By this means it is hoped that the value, from the trade point of view, of weak wheats, such as English wheat, may be increased. Many of these substances, in addition to being "improvers" in the above sense, stimulate the activity of yeast in bread-making.

A process is in use whereby solutions of acid potassium phosphate, diastase and other substances may be sprayed on the flour during or after milling by means of an apparatus similar to that already mentioned for spraying flour with water. The process is considered specially suitable for country millers who have ready access to English wheat, as it is said to enable them to mill a mixture containing a large proportion of English wheat and a correspondingly small amount of strong foreign wheat. It is said, however, that considerable skill and judgment is required in order to obtain good results, and for this amongst other reasons the process has hardly if at all been adopted by English millers. Flour from durum wheat is stated not to be improved.

A certain chemical manufacturer proposes to supply millers and bakers with a mixture of acid potassium phosphate and wheat diastase to be added to flour in the proportion of one or two pounds of the mixture to each sack (280 lbs.) of flour. It is stated that experiments with the above mixture have shown that the flour from certain wheats can be greatly improved as regards strength and water-absorbing capacity.

Another "improver" has lately been introduced. It consists of acid potassium and magnesium phosphates mixed with flour; the mixture

contains phosphates and flour in about equal proportions. The potassium and magnesium phosphates are mixed in the proportion in which they occur in the ash of wheat. The manufacturer recommends that 1 to  $1\frac{1}{2}$  lbs. of the "improver" should be added to each sack of flour (280 lbs.). He considers that this preparation is most effective as an improver. As an inducement to use this preparation, it is represented to the miller that he can use a larger proportion of cheap wheats, and to the baker that as many as 10 to 14 extra 2 lb. loaves can be obtained from a sack of flour by its use.

Manufacturers of acid calcium phosphate have largely advertised this substance to millers and bakers as a desirable flour improver, by means of which the baking qualities and also the colour of the flour may be improved. One such firm issues instructions to add the "improver" at the rate of 2 lbs. per sack of flour (280 lbs.). They guarantee that the flour to which their improver has been added "will be passed as pure wheaten flour by any and every analyst to whom it is submitted for analysis, and that it can be sold as such<sup>1</sup>." They state also that it improves the strength of the flour to the extent of at least 1s. 6d. per sack and the colour by 1s. per sack in value.

Various grades of "acid phosphate" are manufactured—the higher grades by the treatment of bone ash with phosphoric acid, the lower grades by acting on bone ash with sulphuric acid. In the latter case the "improver" may contain 50 per cent. or more of calcium sulphate and an appreciable amount of arsenic. A sample of "improver," recently examined by the public analyst for Kingston-on-Hull, was found to contain 0·07 grain of arsenic per pound, which is seven times the maximum limit suggested by the Royal Commission on Arsenical Poisoning<sup>2</sup>. Both high and low grades of acid calcium phosphate are being offered as flour improvers.

Many millers have experimented with acid calcium phosphate, but it is difficult to ascertain how many of them actually make a practice of adding it to their flour. It is stated that the number is large, and that in many cases the acid phosphate is secretly introduced into the mills for the purpose, or is added on separate premises. In one mill which I visited acid calcium phosphate was being added to, and intimately mixed with, the whole flour output (30 sacks per hour) by means of a specially contrived mixing machine in the proportion of

<sup>1</sup> A method of detecting added acid calcium phosphate in flour and bread has recently been described by G. Curtel, *Annales des Falsifications*, No. 21, 1910, p. 302.

<sup>2</sup> See Final Report of the Royal Commission on Arsenical Poisoning, page 50, 1903 [Cd. 1848].

rather more than  $1\frac{1}{4}$  lbs. per sack of flour (280 lbs.), so that the finished flour contained about 0·45 per cent. of acid calcium phosphate. Other millers whom I visited made a practice of adding acid calcium phosphate to their flour in much the same way.

*Phosphoric Acid.*—The addition of acid in a suitable manner to flour is also said to improve its baking qualities, loaves made from such flour being larger and whiter than those made from untreated flour. The acid preferred is phosphoric acid, and it is claimed that if the process is carefully carried out a great improvement in the baking qualities can be effected in flour made from weak wheat.

In a mill where this treatment is being commercially applied phosphoric acid is allowed to drip slowly into a jet of air and steam; the resulting atomised mixture enters an agitator, where it meets a continuous stream of flour with which it becomes thoroughly incorporated. The introduction of about 0·1 per cent. of phosphoric acid is aimed at, and the difficulty of detecting this by chemical analysis renders the process additionally attractive.

A firm engaged in the chemical industry is interested in an "improver" consisting of semolina which has been treated with phosphoric acid. Thirty parts of phosphoric acid (sp. gr. 1·450) are mixed with 100 parts of semolina, which is then dried and ground. This preparation is directed to be used in the proportion of about  $1\frac{1}{2}$  lbs. to a sack of flour. The firm promise a great increase in the strength and water-absorbing capacity of the flour as a result of this treatment, which, they say, will result in a saving to the miller of 1s. to 1s. 6d. on each sack of flour.

*Other Substances.*—Ozone has been tried for bleaching purposes, but according to Fleurent<sup>(6)</sup> it possesses no bleaching properties, and imparts a disagreeable flavour to the flour. Brahm<sup>(5)</sup> has confirmed this, and has found that the baking qualities of flour are adversely affected by treatment with ozone. Molinari and Soncini<sup>(12)</sup> have shown that ozone attacks and destroys olein, a constituent of the oil of flour.

Sulphur dioxide has been proposed as a bleaching agent for flour, and according to Avery<sup>(3)</sup> and Alway and Gortner<sup>(11)</sup> is effective as far as mere bleaching is concerned. It cannot, however, be used for this purpose, as it destroys the cohesiveness of the gluten.

Flour has also been treated with sulphuryl chloride, sulphur trioxide and chlorine, and other similar mixtures, but I am informed that the results have not been encouraging. Sulphuryl chloride is stated to improve the strength of flour, but the sulphur trioxide and chlorine

mixture is uncertain in action, and is usually without any beneficial effect on the baking qualities of flour. In practice the odour which these substances impart to the flour precludes their use as "improvers."

In some recent patents it is proposed to treat flour with phosphorus trichloride, pentachloride or other halogen compounds of phosphorus, or with a mixture of these and sulphur trioxide, nitric acid, nitrous acid, iodic or other halogen acids; also formic, acetic, propionic or benzoic acids, alcohol, aldehydes, or ketones, with the object of strengthening the flour and improving its baking qualities. It has further been proposed to treat flour with phosphorus pentoxide, phosphorus bisulphide, and phosphorus pentasulphide, and the process has been patented.

Although much experimentation of an empirical kind is proceeding, in the course of which a variety of heterogeneous substances may be added to flour, it may be said that apart from nitrogen peroxide the only substances whose use as yet has been attended with any measure of commercial success are certain acids and salts, more particularly phosphoric acid and phosphates.

#### 6.—BLEACHING AND SO-CALLED FLOUR "IMPROVING" IN RELATION TO PUBLIC HEALTH AND THE GENERAL INTERESTS OF THE CONSUMER.

##### *Bleaching by Nitrogen Peroxide.*

As in the case of preservatives and other deleterious substances added to food in small quantities, great difficulties beset the experimental investigation of the effect of small amounts of nitrogen peroxide, such as are used in bleaching, on the properties of flour and on the health of the consumer.

Although a number of investigations have been undertaken on this subject, the results obtained have been in many respects contradictory and inconclusive. Gluten and bread, prepared from bleached and unbleached flour respectively, have been compared as regards their digestibility by pepsin and trypsin *in vitro* with divergent results. Ladd and Bassett<sup>10</sup> have found that gluten from bleached flour is more slowly digested by these enzymes than is gluten from normal unbleached flour, and a similar but less marked difference has been observed in the case of bread made from bleached and unbleached flour.

Gustav Mann<sup>11</sup> states that gluten from unbleached flour is digested by pepsin about twice as rapidly as gluten from bleached flour

containing 7 parts per million of nitrite-nitrogen. Hale<sup>(7)</sup> has also found that bleaching delays the peptic digestion of flour. Snyder<sup>(5)</sup>, however, found no difference between the behaviour of bleached and unbleached flour towards pepsin.

Halliburton<sup>(8)</sup> has found that traces of nitrates exert a distinctly inhibiting effect on both proteolytic and amylolytic enzymes. One part of sodium nitrite in 8000 was found to inhibit peptic digestion entirely, and one part in 32,000 to decrease the activity of the enzyme to one-seventh of its normal activity. Similar results were obtained in the case of the salivary digestion of starch. From further experiments Halliburton concluded that treatment of a protein with even minute traces of nitrous acid alters it in such a way as to render it less readily susceptible to the action of digestive enzymes. Hale<sup>(7)</sup> has found that sodium nitrite in a dilution of one part in 5000 to 200,000 has a distinct inhibitory action on gastric digestion.

Feeding experiments have also been performed to ascertain whether bleached flour can exert any observable effect upon the living animal. Ladd and White<sup>(11)</sup> administered watery and alcoholic extracts of bleached flour to rabbits by the mouth, and death rapidly supervened. This occurred even though the extracts were neutralised by sodium carbonate before administration. On post-mortem examination the viscera were congested, and the condition of the stomach was such as would result from the administration of an irritant poison. It was suggested that a toxic substance—possibly a diazo-compound—produced in the flour as a result of bleaching was responsible for the death of the rabbits and for the post-mortem appearances. Other observers<sup>(19)(20)</sup> have repeated these experiments, and have failed to obtain the results above described. Wesener and Teller<sup>(19)</sup> have also fed a number of rats for some months upon bread and biscuits made from bleached flour without apparently affecting their health in any way. Hale<sup>(7)</sup> found that alcoholic extracts of overbleached flour were devoid of any marked toxic action when introduced into the stomach.

In this connection, it should be noted that of all biological methods of investigation those involving artificial digestion and feeding experiments require the observance of very special precautions if reliable results are to be obtained. Disregard of such precautions may account for many of the discrepancies which appear in the work done on this subject. Even when due care is exercised, the results obtained from such experiments may be, and frequently are, inconclusive. Numerous disturbing factors inherent in experiments of this kind may

obscure or lead to erroneous interpretation of results which, from the nature of the investigation, often cannot be expected to be marked.

Ladd and White's results, if confirmed, would indicate the existence of a serious danger of acute poisoning from bleached flour. This point was investigated by Dr A. Harden at the Lister Institute. Dr Harden shows that under the conditions in which his experiments were conducted no obvious effects were produced on animals fed with highly bleached flour or with aqueous extracts of such flour.

He also shows that bleaching has a distinctly inhibitory effect on peptic digestion but no observable effect on the pancreatic (proteolytic) digestion of flour. Dr Monier-Williams has demonstrated that sodium nitrite exerts no inhibitory effect on the salivary digestion of starch, but in the case of starch treated with nitrogen peroxide, digestion was greatly retarded. Bleaching was also found to exercise an inhibitory effect on the salivary digestion of flour.

As regards the action of nitrites on the system, it should be remembered that nitrites, when administered as drugs, produce various effects, amongst which disturbance of the heart and vascular system is prominent. The amounts of nitrite introduced by bleached flour would be of a much lower order than those taken when nitrites are given medicinally<sup>1</sup>. Statements have been made, however, by medical practitioners that an appreciable effect may be produced by quite small doses of nitrite: Gustav Mann<sup>(6)</sup> points out that quantities such as half a grain (32 milligrammes) of nitrous acid may be harmful to some individuals. What may be the physiological or pathological effect of ingestion of even smaller doses when taken day by day throughout many months or years it is impossible to say; there is no evidence on the matter, and it would be very difficult to obtain any. It cannot, however, be regarded as desirable that minute doses should be ingested day by day of a drug which in larger single doses has a marked action on the vascular system.

It would appear from experimental and other considerations to which reference has already been made, that, apart from the addition of nitrites, the constitution of flour may be altered by bleaching. Dr Monier-Williams has clearly shown in his report that the oil of flour undergoes a marked change: it has been suggested by Folin<sup>(10)</sup> that nitration of the flour oil, if it occurs, might entail risk to health, since absorption of the oil and its oxidation in the tissue might occur,

<sup>1</sup> The pharmacopoeial dose of sodium nitrite is 1 to 2 grains, and of nitroglycerine (in Liquor Trinitrini)  $\frac{1}{2}$  to  $\frac{1}{4}$  grain.

resulting in the liberation of nitrites. Ozone produced together with nitrogen peroxide in certain bleaching processes also exerts a markedly destructive action on olein, one of the constituents of flour oil. In spite of assertions to the contrary, there seems to be evidence pointing to the possibility of the protein constituents of flour being adversely affected as the result of bleaching. Dr Monier-Williams has shown that the solubility of the proteins and also of the carbohydrates is increased by such treatment. These changes in the oil, the protein and the carbohydrates are, of course, more marked when flour is overbleached, and though overbleaching is not likely nowadays to occur throughout the whole of the flour, local overbleaching may take place, portions of flour adhering to the sides of the agitator and flour spouts may become overbleached and contaminate to some extent, at any rate, the rest of the flour.

Looking to the above considerations, it may be concluded that the alterations in, and the additions to, flour which result from a high degree of bleaching by nitrogen peroxide cannot be regarded as free from risk to the consumer, especially when regard is had to the inhibitory effect of the bleaching agent on digestive processes and enzymes. Even in the case of flour which is bleached to the small extent which is at present ordinarily practised, it would in present knowledge be unwise to conclude that the process is attended by absolute freedom from risk. The fact that bleached flour has been shown to be something more than natural flour, the colour of which has been modified, is also of importance in considering whether bleached flour may properly be represented as genuine flour.

The practice of bleaching being open to these objections, it remains to inquire whether the consumer, who at present is seldom aware that his flour has been bleached, or that his bread is made from bleached flour, can be said to obtain any compensating benefit. To this a negative answer must be given.

Apart from any dietetic considerations a large number of people desire bread of exceptional whiteness, and it is reasonable to suppose that what is demanded by those who prefer such bread is an article made from flour, the whiteness of which is due to its being prepared from specially selected wheats by the elaborate mechanical separation and "purification" of modern milling methods. Few people would carry their approval of whiteness to the extent of requiring naturally dark flour to be chemically treated.

It should be noted in this connection that in some countries where special attention has been given to the bleaching of flour steps have

already been taken to discourage or prohibit the practice. I have already referred to the decisions on the subject by the Board of Food and Drug Inspection of the United States Department of Agriculture. Under the laws against adulteration in certain of the Australian States, the official definitions of what may properly be described as flour exclude flour which has been treated by bleaching, and the official definition of flour recommended by the Departmental Conference on uniform standards for foods and drugs in the Commonwealth and States of Australia (1910) to be applied throughout the Commonwealth, contains a provision expressly excluding the bleaching of flour. In Switzerland the bleaching of flour has been prohibited by Article 61 of the *Loi fédérale sur le commerce des denrées alimentaires et de divers objets usuels* (Du 8 décembre, 1905).

#### *So-called "Flour Improvers."*

The use of these substances is discouraged or prohibited by the official requirements of the countries above referred to, and these articles can hardly be regarded as proper constituents of what is represented to be genuine flour in this country.

Those interested in these preparations advocate their use on the grounds that they add nothing to the flour that is not normally present therein, and that they increase the lightness and improve the quality and appearance of the loaf, and also permit of more loaves being made from a given quantity of flour. The first of these contentions is based on the assumption that phosphorus in flour is present in the form of phosphate, chiefly potassium phosphate. This is not so; it is true only of the ash of flour. A large portion of the phosphorus in flour is present in organic combination, and experimental evidence exists which would seem to indicate that phosphorus in this form may possess a dietetic value quite different from inorganically combined phosphorus. This is recognised by certain millers, and it is suggested that such organic phosphorus compounds may be formed when solutions containing phosphates are intimately mixed with flour in the form of a fine spray from an "atomiser." No evidence is adduced in favour of this contention, and it may suffice to say that the formation of complex organic phosphorus compounds in this way is contrary to experience.

The second advantage claimed, namely, the increased loaf production from a given quantity of flour is one which will appeal only to the miller and baker. The gain in production is due to the increased amount of water which the flour absorbs and to the increase in volume

of the dough, resulting from the improved elasticity of the gluten. This naturally means a diminution in the actual amount of flour in each loaf, and, consequently, in nutritive value, so that the consumer in this respect loses by the treatment.

The protein content of flour is an important matter from the standpoint of nutrition, especially where bread enters largely into a diet. Flour from weak wheats, which are generally poor in gluten, contains less protein than flour from strong wheats which are rich in gluten. But by the use of "improvers" flour from weak wheat is made to simulate flour from a stronger wheat, although as regards protein content it is inferior to the flour which it imitates.

With regard to other substances which have been represented as "improvers," it may be said that the indiscriminate addition of powerful chemical substances such as hydrofluoric acid, phosphorus pentachloride, and the oxides and sulphides of phosphorus to flour is most dangerous.

At the present time many millers are unaware of the nature and composition of the "improvers" which they add to flour, and are content with the assurance of chemical manufacturers that the preparations offered to them constitute, in all respects, desirable additions to flour. If the use of "improvers" became general, there can be little doubt that manufacturers of chemical products would persuade millers to treat their flour with preparations often of a questionable nature, sold under attractive and, possibly, misleading names. There can be no doubt also that competition amongst chemical manufacturers would eventually result in millers being supplied with low grade articles liable to contain dangerous impurities.

It must also be remembered that the addition of foreign substances may not be limited to the miller, but that each person through whose hands flour passes (*e.g.*, the flour factor and the baker), may add to it a different substance or more of what the miller has already added. In this way flour, when it reaches the consumer, may have departed considerably from its original state of purity.

The increasing activity which is now being displayed in the use of different articles as additions to flour must be regarded with considerable apprehension. It does not appear desirable that such an indispensable foodstuff as flour, the purity and wholesomeness of which are of first importance to the community, should be manipulated and treated with foreign substances, the utility of which, from the point of view of the consumer, is more than questionable.

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NOTE:—Numerous papers on wheat and flour were also read at the Seventh International Congress of Applied Chemistry, London, 1909, and at the British Association for the Advancement of Science, Winnipeg, 1909.

## ABSTRACTS OF OFFICIAL PUBLICATIONS, ETC.

### ON THE CHEMICAL CHANGES PRODUCED IN FLOUR BY BLEACHING.

By G. W. MONIER-WILLIAMS, B.A., PH.D., F.I.C.

(*Abstract of a Report to the Local Government Board,  
Food Reports, No. 12.*)

WHEN flour is shaken with air containing a small quantity of nitrogen peroxide gas, the gas is almost immediately absorbed, and the golden-yellow tint of the flour is destroyed. If considerable quantities of the gas are used the flour acquires at the same time a musty odour, which increases with the amount of gas absorbed.

In starting a series of laboratory experiments to determine the changes which may take place in the nature of the flour owing to this treatment, it was necessary to employ a substantial quantity of the gas, considerably more than, as appears from the examination of recent trade samples, is at present employed in British flour mills. Judged by a series of results obtained in the examination of commercial flours for nitrites, it would seem that the bleaching at present adopted in the trade may be effected by gas which on the average is applied only in the proportion of 1 to 2 c.c. of nitrogen peroxide per kilogramme of flour. Inquiries were made at several mills, and the composition of the gas at the inlet and outlet of the agitator where the bleaching takes place was ascertained by analysis in several instances. No data were, however, available as to the quantity of flour treated per unit of gas,

except in one instance. Here the data, while too uncertain to warrant any definite conclusion as to the amount of gas absorbed by the flour, tended generally to show that this was of the same order as that indicated by the results obtained in the examination of commercial flour for nitrites. Considerably larger quantities, such as 20 to 30 c.c. of nitrogen peroxide per kilogramme of flour, appear to have been used in the past, and the possibility of local overbleaching has to be borne in mind.

For the purpose of the inquiry, it seemed desirable to ascertain by laboratory experiment how far the degree of bleaching was affected by a progressive increase in the quantity of nitrogen peroxide used and at what point the maximum bleaching effect could be produced, so that the experimental work, though carried out with larger quantities than are at present used by the millers, might nevertheless be restricted so far as possible to quantities which can be considered to be of possible utility in bleaching processes.

The method adopted in the laboratory for treating flour with accurately measured quantities of nitrogen peroxide gas was as follows:—

200 grms. of a freshly-milled high grade patent flour were introduced into a  $2\frac{1}{2}$  litre vessel fitted with a rubber stopper carrying a capillary tube with glass cock, and a measured quantity of pure nitric oxide gas introduced from a gas-holder. Red fumes of nitrogen peroxide were formed in the vessel, and on shaking for a few minutes were absorbed by the flour. To arrive at the actual quantity of gas taken up by the flour, the vessel was connected with another of equal capacity which had previously been exhausted as completely as possible by a Fleuss pump. Approximately half of the nitrogen peroxide still remaining in the bleaching vessel was in this way collected in the second vessel, in which it was absorbed by shaking with dilute caustic soda solution, and the nitrogen estimated as ammonia by means of Nessler's solution after reduction with aluminium foil and subsequent distillation.

The nitric oxide used was prepared by heating a solution of ferrous sulphate which had previously been saturated with the gas evolved from nitric acid and copper turnings, and contained about 99 % of NO. It was stored in a gas-holder over water, and measured in a mercury burette. In the following table, Column I gives the number of c.c. of nitric oxide used per kilogramme of flour; Column II the corresponding weight of nitrogen, after correcting for atmospheric

pressure (753 mm.), temperature ( $25^{\circ}\text{C}$ .), moisture, and 1 % impurity in the gas; Column III the amount of gas remaining unabsorbed under the conditions of the experiment; and Column IV the nitrogen actually absorbed by the flour as nitrogen peroxide. 200 grammes of flour were used in every case.

NO introduced : c.c. per kgm. flour	NO introduced : grammes Nitrogen per kgm. flour (corrected)	Residual NO <sub>2</sub> : grammes Nitrogen per kgm. flour	NO <sub>2</sub> absorbed : grammes Nitrogen per kgm. flour
5	.00272	.0016	.0011
10	.00544	.0014	.0010
15	.00817	.0018	.0064
20	.01089	.0026	.0083
30	.01633	.0030	.0133
40	.02178	.0026	.0192
60	.03266	.0050	.0277
100	.05445	.0042	.0502
160	.08711	.0030	.0841
230	.12523	.0070	.1182
300	.16335	.0054	.1579

The completeness of the absorption appears to depend upon the length of time the shaking is continued, upon the relative volume of the bleaching vessel compared with the amount of flour present, and upon the amount of bleaching gas used. The absorption is practically complete on prolonged shaking in a small vessel.

The bleached samples were kept in the dark in loosely stoppered glass jars, or in glazed paper bags, and the following series of experiments carried out:—

- (i) Examination of colour by Lovibond's tintometer.
- (ii) Determination of nitrous acid and nitrites by the Griess-Ilosvay method.
- (iii) Determination of nitrogen recoverable as ammonia by reduction of the aqueous extract of the flour with a copper-zinc couple, and subsequent distillation.
- (iv) Determination of the total nitrogen rendered soluble by bleaching, and of the cold water extract of the flour.
- (v) Characteristics of the oil from bleached and unbleached flour.
- (vi) Measurement of the decrease in volume of the air in the bleaching vessel, due to absorption of nitrogen peroxide, with a view to ascertaining whether free nitrogen is produced during bleaching, and tests for the presence of diazo-compounds.
- (vii) Action of dilute nitric acid of various strengths upon pure glutenin and gliadin.

(viii) Salivary digestion of bleached and unbleached flour, and of starch treated with varying amounts of sodium nitrite and nitrogen peroxide.

### I.—COLOUR.

The samples of bleached flour were examined as to colour in a Lovibond tintometer, being pressed into small wooden trays so as to give a flat surface, and then "Pekardised," i.e., dipped carefully into water and slowly withdrawn. All the bleached samples were practically the same colour, the following being the colour equivalents in Lovibond's units of the unbleached flour, the same flour as bleached at the mill, and as bleached in the laboratory.

			Yellow	Red
Unbleached	...	...	1·20	.60
Bleached at mill	...	...	1·05	.57
Bleached in laboratory	...	...	.90	.50

The laboratory bleached samples were kept in the dark in loosely stoppered glass jars, and examined again by the tintometer after the lapse of 17 days, when the colour equivalents in Lovibond units were as follows:—

			Yellow	Red
Unbleached flour	...	...	1·20	.60
5 c.c. NO per kgm. flour	...	...	.90	.50
10 "	"	...	.85	.47
15 "	"	...	.82	.46
20 "	"	...	.80	.46
30 "	"	...	.76	.45
40 "	"	...	.75	.45
60 "	"	...	.75	.45
100 "	"	...	.75	.45
160 "	"	...	.80	.45
230 "	"	...	.85	.45
300 "	"	...	.90	.45

From the above table it will be seen that a considerable change takes place in the colour on keeping. The majority of the bleached samples tend to become still whiter, the maximum of bleaching effect being attained with 30 to 100 c.c. of gas per kilogramme, while with quantities greater than this there is a gradual increase of yellow, but not of red.

## II.—NITROUS ACID AND NITRITES.

A large number of analyses have been published giving the amount of nitrites in the cold water extract from bleached flours (cf. Dr Hamill's report). Extremely small quantities of nitrites can be detected by the Griess-Ilosvay method in which the colour produced with sulphanilic acid and *a*-naphthylamine hydrochloride in acetic acid solution is compared with standards containing known amounts of sodium nitrite. It has been stated that up to a certain point the amount of nitrogen present as nitrite corresponds to the amount of nitrogen in the bleaching gas employed, but that above this point the ratio of nitrite nitrogen to total nitrogen absorbed decreases, becoming still smaller on keeping the flour for several days. With a view to ascertaining the ratio of nitrogen present as nitrous acid or nitrites to the total nitrogen absorbed, and also the rate of disappearance of the nitrites, weighed quantities of the samples were mixed with ten times their weight of well boiled distilled water free from nitrites<sup>1</sup> at the ordinary temperature, allowed to stand for 12 hours with frequent shaking, and centrifuged. Nitrite estimations were then carried out on the clear liquor by the Griess-Ilosvay method. Three series of determinations were made, the first on the samples exactly one hour after bleaching, the second on the same samples after keeping for 20 days, and the third on the same samples after the lapse of 62 days. In the first series the quantity of nitrogen present as nitrites was found to be proportional to the quantity of bleaching gas employed. In the lower members of the series the amount corresponded to about 30% of the total quantity of nitrogen absorbed by the flour, rising to about 40% in the more highly bleached samples. On keeping for 20 days the amount of nitrite present remained approximately the same in the samples bleached with 5 to 60 c.c. of gas per kilogramme, but decreased considerably in the higher members of the series. After keeping for 62 days the nitrites had decreased in amount very greatly, except in the lowest members of the series, while the samples bleached with 230 and 300 c.c. of gas per kilogramme contained slightly less nitrite than those bleached with 100 and 160 c.c. of gas.

<sup>1</sup> It is not easy to obtain distilled water quite free from nitrites. In examining commercial samples of flour it is preferable to use ordinary tap water as this can generally be obtained free from any traces of nitrites.

The following table gives the amount of nitrogen as nitrous acid or nitrites per kilogramme of flour compared with the total quantity of nitrogen absorbed by the flour:—

c.c. Nitric Oxide per kgm. of Hour	N <sub>2</sub> O <sub>4</sub> absorbed, Grammes N per kgm. flour	Nitrogen present as Nitrites or Nitrous Acid		
		One hour after bleaching	20 days after bleaching	62 days after bleaching
0	nil	nil	nil	nil
5	.0011	.0008	4	.0006
10	.0040	.0012	6	.0010
15	.0064	.0020	10	.0014
20	.0083	.0025	12.5	.0024
30	.0133	.0040	20	.0040
40	.0192	.0060	30	.0060
60	.0277	.0090	45	.0084
100	.0502	.0180	90	.0126
160	.0841	.0300	150	.0184
230	.1182	.0450	225	.0200
300	.1579	.0660	330	.0206
				103
				.0035
				17.5

### III.—NITROGEN RECOVERABLE AS AMMONIA BY REDUCTION OF THE AQUEOUS EXTRACT.

When nitrogen peroxide is absorbed by a powder, such as flour, containing a small quantity of moisture, the first result should be combination with the water present to form nitric and nitrous acids. If no further action takes place, the whole of the nitrogen present as nitric and nitrous acids should be recoverable from the aqueous extract of the flour by reduction to ammonia and subsequent distillation, as in the ordinary estimation of nitrates in water analysis. With this object in view weighed quantities of the samples were mixed with ten times their weight of ammonia-free distilled water and thoroughly shaken for several hours in a rotary shaking apparatus. They were then centrifuged as clear as possible, and 200 c.c., corresponding to 20 grammes of flour, treated with a Gladstone-Tribe copper-zinc couple in a stoppered bottle at 37° C., 1 c.c. of toluol being added to prevent decomposition<sup>1</sup>. The couples were prepared by immersing a piece of

<sup>1</sup> In this connection it is interesting to note that bleaching may exercise a considerable preservative action on flour. In a series of extracts containing no toluol the unbleached or slightly bleached samples became sour very much sooner than those which had been highly bleached. (Cf. E. Fléurent, *Bull. Soc. Chim.* [iii.], xxxv. pp. 381-396. 1906.)

sheet zinc measuring  $12 \times 5$  cm. in a 3% copper sulphate solution for 15 minutes, and afterwards washing in distilled water. The bottles were allowed to remain in the incubator until the liquid ceased to give a reaction for nitrites, generally about one or two days. The contents were then diluted to 500 c.c. with ammonia-free distilled water, one gramme of calcium carbonate added, and the ammonia distilled off and estimated colorimetrically by Nessler's solution. Unbleached flour naturally yields a small quantity of ammonia on reduction in this way<sup>1</sup>, and accordingly a blank experiment was always carried out with the unbleached flour under exactly the same conditions, and the amount of ammonia obtained subtracted from that obtained from the bleached samples. It was found that calcium carbonate will liberate the whole of the ammonia from very dilute solutions of ammonium salts on boiling, without acting on the proteins in solution, whereas if a stronger alkali is used a considerable quantity of ammonia is formed by protein decomposition. The following table gives the amount of nitrogen recovered as ammonia compared with the nitrogen actually absorbed by the flour as  $\text{NO}_2$  :—

c.c. Nitric Oxide per kgn. flour	NO <sub>2</sub> absorbed, grammes N per kgn. flour	Nitrogen recovered as Ammonia ; Grammes Nitrogen per kgn. flour			
		One hour after bleaching		22 days after bleaching	
		Total quantity	Subtracting blank	Total quantity	Subtracting blank
0	—	.027	nil	.028	nil
5	.0011	.028	.001	.028	nil
10	.0040	.029	.002	.028	nil
15	.0064	.030	.003	.028	nil
20	.0083	.032	.005	.028	nil
30	.0133	.036	.009	.030	.002
40	.0192	.038	.011	.034	.006
60	.0277	.044	.017	.038	.010
100	.0502	.058	.031	.044	.016
160	.0841	.076	.049	.056	.028
230	.1182	.096	.069	.068	.040
300	.1579	.124	.097	.080	.052

It will be seen from the above results, that the nitrogen recoverable as ammonia is approximately proportional to the total nitrogen absorbed as  $\text{NO}_2$  and amounts, in the case of the samples examined soon after bleaching, to about 60% of the total nitrogen absorbed, falling to about 33% after the lapse of 22 days.

The error of experiment is of course relatively great, especially in the lower members of the series, but the results indicate that the

<sup>1</sup> Cf. J. E. Purvis and R. M. Courtauld, *Proc. Camb. Phil. Soc.* xiv. pp. 441-446. 1908.

greater part of the nitrogen peroxide absorbed by the flour can be recovered as ammonia by reduction of the aqueous extract, and may therefore be assumed to be present as nitric and nitrous acids or as nitrates and nitrites. The nitric acid seems to disappear more quickly than the nitrous acid, since after keeping the flour 22 days the nitrogen recovered as ammonia is approximately equal to that present as nitrous acid or nitrites, except in the case of the three most highly bleached samples. As will be shown later, the oil of the flour absorbs about 6 to 7% of the nitrogen peroxide added, so that somewhat over 30% of the gas remains to be accounted for. This may possibly be present as nitric or nitrous acid in combination with, or mechanically "adsorbed" by, the gluten or starch of the flour, and so escape extraction with cold water. The quantity of water used for extraction did not appear to affect the results. Flour bleached with 300 c.c. of gas per kilogramme was extracted with 100, 200, 400 and 800 c.c. of water per 10 grammes of flour, and the extracts treated with copper-zinc couples, blank experiments being carried out in the same way on the unbleached flour. The amount of ammonia obtained increased with the dilution, but the difference, representing the excess of ammonia due to bleaching, was approximately the same in all cases as the following table shows:—

Flour bleached with 300 c.c. NO per kilogramme, corresponding to an actual absorption of 0·1579 grms. N per kilogramme:—

Number of grammes flour corresponding to one litre extract	Ammonia from bleached flour, grammes N per kgm.	Ammonia from unbleached flour, grammes N per kgm.	Difference, grammes N per kgm.
100	·130	·056	·074
50	·160	·075	·085
25	·176	·088	·088
12·5	·208	·128	·080

The same flour was also extracted in 10% solution immediately after bleaching and compared with the sample extracted one hour after bleaching, the same amount of ammonia being obtained in both cases.

#### IV.—TOTAL NITROGEN RENDERED SOLUBLE BY BLEACHING, AND COLD WATER EXTRACT OF THE FLOUR.

During the reduction of the aqueous extracts by the copper-zinc couple it was noticed that a white precipitate was formed in the liquid, the solution becoming quite opaque, and that this precipitate increased considerably with the amount of gas employed for bleaching. This appeared to be due to the gradual precipitation of proteins by the

ammonia formed in the solution, and it seemed desirable to ascertain the effect of bleaching on the solubility of the proteins of flour. Estimations of total soluble nitrogen and soluble extract were therefore carried out on all the samples. Weighed quantities of bleached flours were shaken with ten times their weight of water, with addition of toluol, in a rotary shaking machine on two or three successive days at the ordinary temperature, and the extracts obtained as clear as possible by means of a centrifuge. In these extracts, total nitrogen was estimated on 40 c.c. by Kjeldahl's method and total solids by evaporating 40 c.c. in nickel basins on the water bath and drying in a water-oven at 96° C.

Two series of estimations were done, the first on a patent flour bleached 21 days after milling, the extracts being made one hour after bleaching, and the second series on the same flour bleached the day after milling, and extracted 16 days after bleaching. Blank estimations were in both cases carried out on the unbleached flour. The results are given in the following table:—

c.c. Nitric Oxide per kgm. flour	Flour bleached three weeks after milling and extracted one hour after bleaching			Flour bleached one day after milling and extracted 16 days after bleaching		
	Total soluble Nitrogen, grammes per kgm.	Soluble Proteins (N × 5·7), grammes per kgm.	Extract, grammes per kgm.	Total soluble Nitrogen, grammes per kgm.	Soluble Proteins (N × 5·7), grammes per kgm.	Extract, grammes per kgm.
0	4·16	23·7	81·3	3·01	17·1	96·0
5	4·16	23·7	81·5	3·13	17·8	96·3
10	4·27	24·3	81·3	3·15	18·0	96·6
15	4·10	23·4	80·6	2·99	17·0	96·9
20	4·27	24·3	83·1	3·19	18·2	97·8
30	4·31	24·6	83·9	3·32	18·9	99·0
40	4·37	24·9	85·2	3·26	18·6	101·4
60	4·55	25·9	88·0	3·29	18·8	105·1
100	5·25	29·9	94·0	3·50	20·0	110·6
160	6·37	36·3	103·9	4·88	27·8	127·0
230	7·84	44·7	118·9	5·98	34·1	138·9
300	8·82	50·3	127·6	7·31	41·7	145·6

From the above it will be seen that although in the lowest members of the series an increase in soluble nitrogen and total extract can hardly be detected, in the more highly bleached samples a very large amount of nitrogen is rendered soluble<sup>1</sup>, far more than can be accounted for by

<sup>1</sup> The action of extremely dilute acids and alkalis on the gluten of flour has been investigated by Wood and Hardy (*Proc. Roy. Soc.* 1909, Series B, lxxxi, pp. 38-43), and will be referred to again when dealing with the action of dilute nitric acid on pure glutenin and gliadin.

the nitrogen introduced into the flour during bleaching. Moreover the increase in total soluble matter is greater than the increase in soluble proteins, assuming the nitrogen to be present in the form of protein.

Subsequent experiments on the solubility of the glutenin and gliadin of wheat flour in very dilute nitric acid confirmed the above results as regards the increase of soluble proteins. The aqueous extracts were therefore further investigated in order to account, if possible, for the still greater increase of total soluble matter.

Two of the extracts, one from the unbleached flour and one from the same flour bleached with 300 c.c. of gas per kilogramme and extracted 16 days after bleaching, were treated with mercuric chloride and alumina cream to remove proteins, the mercury removed from the filtered liquids by sulphuretted hydrogen, and the resulting solutions, measuring 75 c.c., boiled for 15 minutes with  $12\frac{1}{2}$  c.c. of concentrated hydrochloric acid. They were then cooled, neutralised, made up to 100 c.c., and 10 c.c. titrated for dextrose by Ivar Bang's method (*Biochem. Zeitsch.*, II. p. 271, 1906). The amount of dextrose found corresponded in the unbleached sample to 54·0 and in the bleached sample to 81·5 grammes per kilogramme of flour. Calculated to dextrin this gives 48·6 and 73·3 grammes dextrin per kilogramme respectively.

It appears therefore that a considerable amount of carbohydrate as well as protein is rendered soluble by bleaching. The following table shows the relation of carbohydrate to protein in the extract:—

	Total soluble matter, grammes per kgm. flour	Soluble proteins, grammes per kgm. flour	Soluble carbohydrate as Dextrin, grammes per kgm. flour	Dextrin + protein, grammes per kgm.
Unbleached flour	96·0	17·1	48·6	65·7
Bleached flour	145·6	41·7	73·3	115·0
Difference	49·6	24·6	24·7	49·3

It has been pointed out (Allen, *Commercial Organic Analysis*, 1898, iv. p. 79) that a constant ratio exists between the proportion of soluble proteins and soluble carbohydrates in flour. From the above results it appears that the actual increase in soluble carbohydrate due to bleaching is approximately equal to the increase in soluble protein, while the original ratio of soluble carbohydrate to soluble protein decreases. The amount of soluble matter other than carbohydrate and protein does not appear to be altered by bleaching, being in the above case 30·3 grammes per kilogramme for the unbleached, and 30·6 grammes per kilogramme for the bleached flour.

V.—CHARACTERISTICS OF THE OIL FROM BLEACHED AND UNBLEACHED FLOUR.

One kilogramme of a patent flour was extracted with petroleum ether and the solvent evaporated off on a water bath in a current of carbonic acid gas in order to avoid oxidation of the oil. The oil was also extracted in the same way from samples of the same flour which had been bleached with 150 and 300 c.c. of gas respectively per kilogramme, the extraction being made one hour after bleaching. The oil from the unbleached sample was opaque and golden-yellow in colour, and gave an intense golden-yellow solution in petroleum ether and chloroform, while the oils from the bleached samples were reddish-yellow and gave an almost colourless solution in the same solvents. The following constants were obtained for the oils:—

c.c. NO <sub>2</sub> per kgm. flour	Iodine value (Hubl)	Saponification value (Kottstorfer)	Saponification Index (C.I.) <sup>1</sup>	Total Nitrogen
0	{ 102·2   101·8	170·0	303·6	1·12 per cent.
150	{ 98·7   100·3	170·1	303·8	1·20 ,,
300	{ 100·6   101·2	170·6	304·7	1·23 ,,

The total nitrogen was estimated by Jodlbauer's modification of Kjeldahl's method, using zinc dust and salicylic acid.

From the above it appears that if the oil is examined immediately after bleaching, no alteration except as regards colour, and a slight increase in the nitrogen content, can be detected. It being possible, however, that if any action on the oil takes place, it would be more evident after the lapse of several days from the time of bleaching, another sample of flour was bleached and allowed to stand in closed glass jars in the dark for seven days. The flour yielded 0·8% of oil (petroleum ether extract). 2000 grammes were bleached with 100 and 300 c.c. of gas per kilogramme respectively, and the oil extracted with petroleum ether. The solvent was evaporated off on the water bath under diminished pressure in a current of carbonic acid gas.

The oils showed the same characteristics in regard to colour as in the previous case, that from the unbleached flour being golden-yellow,

<sup>1</sup> The letters C.I. indicate the methods of expression recommended by the International Conference for the Unification of Methods of Analysis (Paris, 1910).

that from the flour bleached with 100 c.c. per kilogramme reddish-yellow, and that from the more highly bleached sample dark reddish-yellow. The viscosity had been increased by bleaching, especially with the more highly bleached sample which was exceedingly viscous.

As many of the constants as possible were determined with the limited quantity of material at disposal, and very great differences were observed, especially with the oil from the highly bleached sample. The following numbers were obtained on analysis of the oils:—

	Unbleached flour	100 c.c. NO <sub>2</sub> per kgm. flour	300 c.c. NO <sub>2</sub> per kgm. flour
Specific gravity $\frac{20^\circ}{15^\circ}$ C.	0·946	0·952	0·985
Iodine value (Hübl)	96·7	92·6	60·7
	97·1	94·5	61·9
Saponification value (Köttstorfer)	158·1	159·4	166·3
Saponification index (C.I.) <sup>1</sup>	282·4	284·7	297·0
Solidifying point of fatty acids	26·5° C.	28·0° C.	32·4° C.
Reichert-Wollny number	0·8	0·9	3·5
Acid value	12·5	11·2	12·4
Acidity in e.c. $\frac{N}{I}$ alkali per 100 grammes			
oil (C.I.) <sup>1</sup>	22·3	20·0	22·1
Total nitrogen	0·83 %	0·89 %	0·96 %

It is evident that a very considerable oxidation of the oil takes place when flour is highly bleached, although it is possible that the effects produced may be to a certain extent due to the "elaidin" reaction having taken place under the influence of the nitrous acid present. The length of time that the flour is kept after bleaching appears to be an important factor in the effect produced on the oil, and it is possible that even with slightly bleached flours the characteristics of the oil would be gradually altered by long storage. The increase in nitrogen corresponds roughly to about 6% of the total nitrogen absorbed by the flour in the form of nitrogen peroxide, and the same increase is also apparent in the oil extracted immediately after the flour has been bleached, although oxidation has not yet taken place<sup>2</sup>.

<sup>1</sup> The letters C.I. indicate the methods of expression recommended by the International Conference for the Unification of Methods of Analysis (Paris, 1910).

<sup>2</sup> Egoroff (*Journ. Russ. Phys. Chem. Soc.* 1903, xxxv. pp. 973-997), has shown that small quantities of nitrogen peroxide act on oleic acid giving an additive product, and it is only where this latter is present in a certain proportion, for the formation of which a certain minimum quantity of nitrogen peroxide is required, that the isomeric change of oleic into elaidic acid can take place.

VI.—CHANGE OF VOLUME IN AIR CONTAINING NITROGEN PEROXIDE  
DUE TO ABSORPTION OF THE GAS BY FLOUR, AND TESTS FOR  
THE PRESENCE OF DIAZO-COMPOUNDS.

In view of the assertion that free nitrogen may be produced by the action of nitrogen peroxide on flour, the following experiment was devised with a view to ascertaining whether this is in fact the case.

200 grammes of flour were introduced into a glass globe of 1500 c.c. capacity, fitted with a capillary tube and glass cock at one side, and a wide tube with glass cock at the other, and the globe was placed in a water thermostat which could be kept constant to within 0·2° C. The capillary tube was connected with a water manometer, and the vessel allowed to remain in the thermostat until equilibrium was attained at atmospheric pressure. It was then removed from the thermostat, 60 c.c. of pure nitric oxide introduced, and the vessel well shaken. It was then replaced in the thermostat and allowed to attain equilibrium. On connecting again with the manometer a decrease in pressure was observed owing to the absorption of the nitrogen peroxide. Air was then introduced from a gas burette until the whole was again at atmospheric pressure.

Since 60 c.c. of nitric oxide combine with 30 c.c. of oxygen to give 60 c.c. of nitrogen peroxide, there will be a diminution in the volume of the whole system of 30 c.c. if the whole of the nitrogen peroxide is absorbed by the flour. If nitrogen or any other gas is evolved during bleaching, the diminution in volume will be less than 30 c.c. and less than 30 c.c. of air will have to be added to bring the whole to atmospheric pressure. The error due to changes in temperature may be considerable, but by keeping the thermostat constant to within 0·2° C., this error will not amount under the conditions of the experiment to more than 1 c.c. If the production of nitrogen were an essential part of the reaction the quantity produced would probably be considerably more than this when 60 c.c. of bleaching gas are employed. In no case could an evolution of nitrogen be detected. It being conceivable that an actual absorption of free oxygen by the oil of the flour might take place under the influence of the bleaching gas, and thereby mask any evolution of nitrogen, the experiment was repeated with fat-free flour, but the same result was obtained as with ordinary flour.

The nitrogen peroxide remaining unabsorbed in a globe of 1500 c.c. containing 200 grammes of flour is so small that it may be neglected.

The following were the quantities of air which had to be added to bring the vessel to atmospheric pressure:—

Ordinary flour	Fat-free flour
(i) 29·2 c.c.	(i) 29·6 c.c.
(ii) 30·2 c.c.	(ii) 29·9 c.c.
(iii) 30·2 c.c.	

The above experiment shows, therefore, that no appreciable quantity of nitrogen is evolved during bleaching, provided that there is no simultaneous absorption of free oxygen by any constituent of the flour other than the fat, which would compensate for the change in volume due to the production of nitrogen.

In view of the possibility of diazo-compounds being formed by the action of the nitrogen peroxide on the proteins of the flour, samples of bleached and unbleached flour were subjected to the tests given by Treves and Pellizza (*Atti R. Accad. Sci. Torino*, XXXIX, pp. 429-434, 1904). These observers found that certain proteins react with nitrous acid to give diazo-compounds, which give brownish-red azo-compounds with alkaline  $\beta$ -naphthol and salicylic acid. No difference in colour, however, could be observed between highly bleached and unbleached flour when treated with these reagents.

#### VII.—ACTION OF DILUTE NITRIC ACID UPON PURE GLUTENIN AND GLIADIN.

In order to ascertain, if possible, the nature of the protein rendered soluble by bleaching, and to study the effect of dilute nitric acid solutions upon the proteins of flour, it appeared essential to work upon the pure substances. For this purpose a considerable quantity of glutenin and gliadin was prepared for me by Dr S. B. Schryver, to whom I am much indebted for advice in connection with this part of the inquiry. The proteins were prepared by Osborne's method from a large quantity of flour, and were obtained as dry white powders, which could be kept without change in stoppered vessels over calcium chloride.

Weighed quantities of each protein were allowed to remain in contact with excess of  $\frac{N}{10}$ ,  $\frac{N}{100}$  and  $\frac{N}{1000}$  nitric acid at the laboratory temperature for several days, the vessels being frequently shaken, and a few drops of toluol added to each to prevent decomposition. The amount of protein dissolved was estimated by evaporating a part of the solution to dryness on the water bath, with the addition of ammonia to neutralise the free nitric acid, and subtracting from the weight of the residue the weight of ammonium nitrate corresponding to the nitric

acid originally present. The following were the quantities of protein dissolved, in grammes per 100 c.c. of solution:—

	Gli tenin	Gliadin
Distilled water	0·0244	0·1056
N $\frac{N}{100}$ nitric acid	0·0788	0·7388
N $\frac{N}{100}$ nitric acid	0·1028	5·1470
N $\frac{N}{10}$ nitric acid	0·0296	0·0032

These results are in agreement with those of Wood and Hardy (*Proc. Roy. Soc.*, 1909, Series B, LXXXI. pp. 38–43), who investigated the action of dilute acids, alkalies and salts upon the physical state of gluten. They found for nitric acid a “critical concentration” of  $\frac{3N}{100}$  at which gluten just retains its coherence. Where gluten is treated with nitric acid below this “critical concentration” it loses its coherence and is dispersed to form an opaque colloidal solution or hydrosol.

The physical properties of gluten are largely due to the gliadin it contains, and the relations of gliadin to acid and alkali are the same as those of gluten.

It appears, therefore, that gliadin forms a hydrosol with extreme ease under the influence of nitric acid, especially at or near a concentration of  $\frac{N}{100}$ , while glutenin is hardly affected.

In the case of the majority of commercially bleached flours, which may contain very minute quantities of nitric acid, the effect on the gliadin will no doubt depend on the amount of neutral or alkaline salts that the flour may contain. Wood and Hardy (*loc. cit.*) have shown that the dispersion of gluten may be completely prevented by the addition of salts.

It was shown above that by reduction of the aqueous extract of a bleached flour with a copper-zinc couple, only 60 % of the nitrogen absorbed during bleaching could be recovered as ammonia, while 6 to 7 % is absorbed by the fat, leaving about 30 % still unaccounted for. It seemed possible that the nitrogen unaccounted for might be present in the form of nitric acid in combination with, or “adsorbed” by, the glutenin or gliadin of the flour. Some experiments were therefore carried out on the reduction of dilute nitric acid solutions containing glutenin and gliadin with a copper-zinc couple, with a view to ascertaining whether the whole of the nitrogen of the nitric acid is recoverable as ammonia under these conditions.

Weighed quantities of each protein were thoroughly mixed with measured amounts of nitric acid of different strengths, and allowed to stand a certain time. The mixtures were then made up to 200 c.c. with ammonia-free distilled water. The liquids were centrifuged and treated with a copper-zinc couple in an incubator at 37° C., a few drops of toluol being added, until no further reaction for nitrites was given. They were then diluted to 500 c.c., one gramme of calcium carbonate added, and the ammonia distilled off and estimated by Nessler's solution. A blank experiment was made at the same time on nitric acid alone.

It was found that with gliadin the whole of the nitric acid could be recovered as ammonia, provided that the amount of gliadin present was not greater than could pass completely into solution. If a greater quantity of gliadin was present than could be dissolved in the liquid, the undissolved portion formed a semi-fluid mass at the bottom of the vessel and retained a part of the nitric acid. In the case of glutenin, which is only very slightly soluble, considerable "adsorption" of nitric acid appears to take place when the protein is treated with  $\frac{N}{100}$  nitric acid, and slight adsorption with  $\frac{N}{1000}$  nitric acid, but none at all with  $\frac{N}{10}$  acid. The following table shows the quantities of protein and nitric acid taken, and the amount of nitrogen recovered as ammonia from the solutions:—

			Duration of contact of protein and acid	Grammes of nitrogen added as nitric acid	Grammes of nitrogen recovered as ammonia
2 e.e. $HNO_3$	$\frac{N}{10}$	...	—	.0028	.0028
2 grammes gliadin + 2 e.e. $HNO_3$	$\frac{N}{10}$	...	2 days	.0028	.0027
" "	" "	...	20 "	.0028	.0028
5 grammes gliadin + 20 e.e. $HNO_3$	$\frac{N}{100}$	...	3 "	.0028	.0015
1 gramme gliadin + 20 e.e. $HNO_3$	$\frac{N}{100}$	...	2 "	.0028	.0028
1 gramme gliadin + 200 e.e. $HNO_3$	$\frac{N}{1000}$	...	2 "	.0028	.0028
2 grammes glutenin + 2 e.e. $HNO_3$	$\frac{N}{10}$	...	2 "	.0028	.0026
" "	" "	...	20 "	.0028	.0028
5 grammes glutenin + 20 e.e. $HNO_3$	$\frac{N}{100}$	...	3 "	.0028	.0015
2 grammes glutenin + 20 e.e. $HNO_3$	$\frac{N}{100}$	...	2 "	.0028	.0016
2 grammes glutenin + 200 e.e. $HNO_3$	$\frac{N}{1000}$	...	2 "	.0028	.0024
1 gramme gliadin } + 2 e.e. $HNO_3$	$\frac{N}{10}$	...	2 "	.0028	.0028
1 gramme glutenin }	" "	..."	20 "	.0028	.0028

It would appear, therefore, that both gliadin and glutenin are able under certain conditions to adsorb nitric acid from dilute solution, and this may to some extent account for the failure to recover the whole of the nitrogen introduced during bleaching, by reduction of the aqueous extract of the flour.

### VIII.—DIGESTION EXPERIMENTS. EFFECT OF NITROGEN PEROXIDE AND OF SODIUM NITRITE ON THE SALIVARY DIGESTION OF STARCH AND FLOUR.

#### (i) *Effect of Sodium Nitrite on the Salivary Digestion of Starch.*

It has been stated by Halliburton (*Journal of Hygiene*, ix. p. 170, 1909), that small quantities of sodium nitrite exercise an inhibitory effect on the salivary digestion of starch, as measured by the time required to reach the achromic point when tested with a solution of iodine. I have not, however, been able to confirm this, and have found on the contrary that within certain limits the addition of minute quantities of sodium nitrite slightly accelerates the action of the saliva, but that a marked inhibition results when the starch has been previously treated with nitrogen peroxide gas.

A 1% solution of soluble starch in distilled water was prepared and to quantities of 200 c.c. were added ·04, ·02, ·01, ·005 and 0 grammes of sodium nitrite respectively. The solutions were warmed to 37°C., and 20 c.c. of a 10% solution of saliva added. They were then placed in an incubator at 37°C. and quantities of 20 c.c. withdrawn from each at intervals and immediately added to 30 c.c. of mixed Fehling's solution + 30 c.c. of water. The tubes containing the Fehling's solution were then heated for exactly 20 minutes in a boiling water bath, the liquids filtered, and the precipitates ignited and weighed as cupric oxide. The following table gives the weights of cupric oxide obtained:—

Grammes NaNO <sub>2</sub> per 100 c.c. starch paste	CuO obtained from 20 c.c. after time interval of					
	11 mins.	25 mins.	45 mins.	70 mins.	100 mins.	140 mins.
Nil	·0504	·0861	·1170	·1305	·1408	·1483
·0025	·0508	·0888	·1208	·1347	·1436	·1508
·005	·0525	·0899	·1229	·1345	·1431	·1508
·01	·0554	·0935	·1252	·1364	·1471	·1524
·02	·0601	·0969	·1306	·1404	·1484	·1533

A blank estimation on 20 c.c. of starch paste + the equivalent quantity of saliva solution gave 0·0150 gramme of cupric oxide.

It will be seen from the above results that small quantities of sodium nitrite tend to accelerate the action of the ptyalin of saliva upon starch, the acceleration increasing with the quantity of sodium nitrite present<sup>1</sup>. When larger quantities of sodium nitrite are added, the acceleration increases up to a certain point, after which the rate of digestion becomes irregular. 20 c.c. of a 1% starch solution + 2 c.c. of a 10% solution of saliva were treated with quantities of sodium nitrite varying from '01 gramme to '175 gramme per 100 c.c. of starch solution, and the amount of copper reduced by the solution after one-and-a-half hours determined as in the preceding experiment. The following were the weights of cupric oxide obtained:—

Grammes NaNO <sub>2</sub> per 100 c.c. starch paste	Grammes CuO obtained
0	·0924
·010	·1007
·025	·1067
·040	·1075
·055	·1111
·070	·1100
·085	·1083
·100	·1060
·115	·0900
·130	·0909
·145	·1059
·160	·1037
·175	·0993

#### (ii) *Effect of Nitrogen Peroxide on the Salivary Digestion of Starch.*

Five grammes of soluble starch were introduced into a glass vessel with ground-in stopper carrying a capillary tube with glass cock, and treated with varying quantities of nitrogen peroxide. One per cent. solutions of the starch were then digested with saliva as in the previous experiments for one-and-a-half hours at 37°C., and the weight of copper reduced by 20 c.c. determined. The solutions were slightly acid to litmus and there was very marked inhibition in all of them; practically no starch hydrolysis occurred in three of the samples. The weights of cupric oxide obtained were as follows:—

c.c. NO <sub>2</sub> per 100 c.c. starch paste	Equivalent in grammes NaNO <sub>2</sub> (corrected)	CuO obtained from 20 c.c. of solution
0	0	·1447
0·8	·0025	·1098
1·6	·005	·0223
3·2	·01	·0202
6·4	·02	·0234

<sup>1</sup> Cf. Wohlgemuth (1908), *Biochem. Zeitschr.* ix, pp. 10-43.

(iii) *Salivary Digestion of Bleached and Unbleached Flour.*

The effect of bleaching upon the salivary digestion of flour was investigated by a polarimetric method. Ten grammes of flour were thoroughly mixed with 200 c.c. of water, and heated on the water bath with constant shaking to 85° C. to gelatinise the starch. The mixture was then cooled to 37° C., and portions of 20 c.c. measured into a series of stoppered bottles, which were placed in an incubator at 37° C. Five c.c. of a 4% solution of saliva were added to each, and at different intervals the bottles were removed from the incubator, and 20 c.c. of a solution of tannic acid added. The tannic acid solution was prepared by dissolving 10 grammes of the acid in 475 c.c. of 50% alcohol and adding 20 c.c. of 25% acetic acid with 1 c.c. of concentrated hydrochloric acid.

After standing overnight the liquids were filtered, and the filtrates examined in a polarimeter. The tannin precipitates the proteins and the unaltered starch and yields a perfectly clear filtrate containing the soluble carbohydrates produced by the action of the ptyalin. The rotation rises at first rapidly owing to the formation of soluble dextrins, and if the reaction is carried to completion a decrease in rotation should subsequently take place owing to the conversion of the dextrins into maltose. Under the particular conditions of the experiment an actual decrease in rotation was not observed. As will be seen from the table below, bleaching exercises a marked inhibitory action on the formation of dextrins. In the bleached samples the rotation appears to reach a higher value eventually than in the unbleached sample. This seems to indicate that the simultaneous formation of maltose with consequent diminution in rotation takes place more rapidly in the unbleached than in the bleached samples.

The actual rotations observed were as follows (200 mm. tube):—

Time in minutes	Unbleached flour	Flour bleached with 100 c.c. N <sub>2</sub> O <sub>5</sub> per kgm.	Flour bleached with 300 c.c. N <sub>2</sub> O <sub>5</sub> per kgm.
0	+0·64°	+0·64°	+0·67°
1	+1·05°	+0·94°	+0·89°
2	+1·27°	+1·18°	+1·03°
3	+1·45°	+1·37°	+1·15°
4	+1·64°	+1·55°	+1·34°
6	+1·89°	+1·86°	+1·55°
9	+2·05°	+2·10°	+1·76°
12	+2·15°	+2·20°	+1·94°
15	+2·23°	+2·35°	+2·10°
18	—	+2·40°	+2·18°
22	+2·33°	+2·48°	+2·30°

## SUMMARY OF RESULTS.

The action of air containing nitrogen peroxide upon flour, in quantities up to 300 c.c. of nitrogen peroxide to one kilogramme of flour, may be summarised as follows:—

I. The golden-yellow tint of the flour is destroyed. Immediately after bleaching no difference in tint due to excess of the bleaching agent could be observed with Lovibond's tintometer, but on keeping for several days the more highly bleached samples became decidedly yellow, while those treated with 30 to 100 c.c. of nitrogen peroxide per kilogramme became still whiter, the maximum of bleaching effect being attained within these limits.

II. The amount of nitrous acid or nitrites present in a freshly bleached flour is approximately proportional to the amount of nitrogen peroxide employed, and corresponds to about 30% of the total nitrogen absorbed, rising to 40% in the more highly bleached samples. After the lapse of several days, the proportion of nitrites present decreases considerably in the higher concentrations, but remains very nearly the same in the more slightly bleached samples.

III. Approximately 60% of the total nitrogen introduced as nitrogen peroxide into the flour during bleaching can be recovered as ammonia a short time after bleaching by reducing the aqueous extract of the flour with a copper-zinc couple, and may be assumed to be present in the flour as nitric and nitrous acids or as nitrates and nitrites. After keeping the bleached flour for some days the amount of nitric acid extracted with cold water decreases. Experiments with pure glutenin and gliadin indicated that in certain circumstances nitric acid may be withdrawn from solution or "adsorbed" by these proteins.

IV. In highly bleached flour a considerable increase in the amounts of soluble proteins and soluble carbohydrates takes place. If one kilogramme of flour is bleached with 300 c.c. of nitrogen peroxide, the amount of soluble nitrogen is doubled. This appears to be due almost entirely to the solubility of gliadin in nitric acid of certain concentrations. The simultaneous increase of soluble carbohydrates would seem to point to an intimate relationship between the gliadin and certain carbohydrates in flour.

V. If highly bleached flour is allowed to stand for some time after bleaching, the oil undergoes very considerable alteration and acquires the characteristics of an oxidised oil. About 6 to 7% of the nitrogen

introduced as nitrogen peroxide during bleaching is absorbed by the oil.

VI. The absorption of nitrogen peroxide by flour does not appear to be accompanied by the production of free nitrogen, nor was any evidence obtained of the formation of diazo-compounds.

VII. Sodium nitrite was found to exert no inhibitory action on the digestion of soluble starch by saliva, but the rate of digestion was greatly retarded if the starch had been previously treated with nitrogen peroxide gas. Bleaching was found to exercise an inhibitory effect on the salivary digestion of flour.

A NOTE ON SOME ATTEMPTS TO CAUSE THE FORMATION  
OF CYTOLYSINS AND PRECIPITINS IN CERTAIN  
INVERTEBRATES.

BY G. HAROLD DREW.

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THE results obtained in these investigations were negative throughout, but perhaps are worth recording as tending to show that the formation of such 'anti-bodies' as cytolsins and precipitins is not a necessary reaction on the part of any living organism to the presence of foreign cells or substances.

If deductions can be made from such purely negative evidence, it would seem that the production of an 'anti-body' is not essentially a function of protoplasm, but that the power of the formation of such protective substances has been developed by higher types of animals in the course of evolution.

The animals used in the experiments were *Arca ponderosa*, *Pecten dislocatus*, *Pecten maximus*, *Pecten opercularis*, *Echinus acutus*, and *Echinus miliaris*.

1. *Experiments on the production of cytolsins.*

In one series of experiments a portion of the testis of *Pecten maximus* was removed, washed in a stream of sterile sea water, and a small piece from the interior dissected out and shaken in a little sterile sea water. A suspension of the sperm was thus obtained, and this was injected by means of a hypodermic syringe into the tissues of *Pecten opercularis*, *Echinus acutus*, and *Echinus miliaris*. In the case of *P. opercularis* the injection was made laterally into the highly vascular adductor muscle: in the case of *E. acutus* and *E. miliaris* a portion of the surface of the shell was sterilised by dropping on a little hydrogen peroxide solution (20 vols.), a small hole was then bored

through the calcareous plates with a drill, and the injection made into the coelomic cavity ; the hole was then sealed by dropping on a little melted shellac. This procedure did not appear to injure the animals, and the seal could always be removed and replaced by fresh shellac when it was wished to make further inoculations into the same animal.

Inoculations of 0·5 c.c., 1 c.c., and 2 c.c. of the suspension of sperm were made at intervals of 2, 4, and 6 days in different series of experiments, and still larger quantities of sperm were used in some of the experiments on the *Echini*. A total of six inoculations were made in each series.

The blood of *P. opercularis* consists of a colourless fluid containing a number of amoeboid corpuscles, which, when the blood is shed, become fixed to each other by their pseudopodial processes, and thus form an agglutinated mass resembling a plasmodium. The clear plasma, which can be obtained by filtering off these corpuscles, is of about the same salinity as the sea water in which the animals live, and contains a coagulable proteid in solution. The coelomic fluid of the *Echini* contains corpuscles of various kinds, some possessing pigment granules, but they can all be removed by shaking the blood, when, as in the case of *Pecten*, they become entangled in a plasmodial mass and settle to the bottom. The plasma contains a rather larger amount of coagulable proteid than that of *Pecten*.

The blood of *P. opercularis*, and the coelomic fluids of the *Echini*, were removed at varying intervals, well shaken to cause the corpuscles to agglutinate, and filtered through glass wool. A little sperm of *P. maximus* was then added to the clear plasma obtained in this way, and 'hanging drop' preparations made of the mixture. Examination under the microscope showed no sign of any cytolytic action, nor did the active movements of the spermatozoa cease sooner than in check preparations made with normal plasma.

Repeated injections of the prepared plasma from the *Echini* and *P. opercularis*, in quantities varying from 1 c.c. to 5 c.c., were made into *P. maximus*, but produced no visible effect on the testis either macro- or microscopically.

Similar but more extensive series of experiments were made by injecting the ripe ova of *P. maximus* into the same animals as were used in the experiments on the injection of sperm, and injections of extracts of the ovary, made by pounding in a mortar with sterile sea water, were also made. Entirely negative results were again obtained.

Further experiments were made with the blood of *Arca ponderosa*, which among invertebrates is peculiarly suited for this work. The blood of this animal is red, the colour being due to the presence of a number of discoidal corpuscles containing haemoglobin. In the fresh condition these corpuscles somewhat resemble those of mammalia, but when fixed and stained they can be seen to possess a three or four lobed nucleus; they also contain a few minute granules of some dark brown substance. They number about 80,000 per c.mm. In addition to these cells a number of colourless amoeboid cells, similar to those found in other Lamellibranchs, are present. The plasma is relatively rich in coagulable proteids, but does not possess the power of clotting. When the blood is withdrawn and allowed to settle, it separates into three layers, at the bottom are the red corpuscles, next is a thin layer of the colourless cells which agglutinate to form a plasmodial mass, and above is a perfectly clear layer of serum having a faint yellowish tinge. If the blood be collected aseptically, and kept in sealed sterile glass tubes at room temperature, the blood cells will remain unaltered, and the masses of white cells retain the power of moving their pseudopodial processes for about six days: after longer intervals (10 to 14 days) under these conditions, haemolysis sets in, the colourless cells disappear, the red corpuscles become much crenated and tend to disintegrate, and a clear solution of their haemoglobin in the serum results. The normal serum, when added to the normal serum of *Pecten dislocatus*, produces a fairly copious precipitate of some proteid substance.

A series of injections of the blood of *Arca ponderosa* was made into the adductor muscle of *Pecten dislocatus* in 0·25 c.c., 0·5 c.c., and 1 c.c. doses, at intervals of 2, 4, and 6 days in different series of experiments. The animals into which 1 c.c. of the blood was injected did not live for more than a few days, and as they became manifestly unhealthy soon after the injection, were discarded: those into which 0·5 c.c. was injected at two day intervals also did not survive more than two or three injections. On the average 1·5 c.c. of blood was about the total maximum that could be given, even in small doses at long intervals.

On withdrawing the blood of the *Pectens* experimented on in this way, and examining it under the microscope, it was seen that most of the injected red corpuscles were very little changed, and many appeared quite normal: some showed a certain amount of crenation, and on fixing and staining showed signs of nuclear degeneration,

and a few were surrounded by white cells which appeared to be exercising a phagocytic action on them. The number of red corpuscles which are destroyed in this way is comparatively small, so that an examination of the blood of a *Pecten* into which 0·25 c.c. of the blood of *Area* has been injected will show the presence of approximately the same number of red corpuscles after six days as immediately after the injection.

The blood of the *Pectens* into which the injections had been made, was removed at varying intervals, filtered, and added to the red corpuscles of *Area* which had been washed free from the plasma with sterile sea water. No haemolysis resulted, and examination under the microscope showed that the red corpuscles were quite unaffected by the treatment.

It must therefore be concluded that no cytolsin had been produced.

## 2. Experiments on the production of precipitins.

In these experiments an attempt was made to cause the formation of a precipitin as a reaction to the injection of egg albumen. For this purpose the egg albumen was diluted with four times its bulk of sterile sea water, well shaken, and filtered: this procedure was necessary as egg albumen normally contains a small amount of some substance which gives a slight floccular precipitate when it is diluted with sea water, and which might be a source of error when testing for the precipitin reaction as it also gives the same precipitate with the normal plasma of the blood of marine invertebrates.

This diluted solution of albumen was injected into all the six species of animals mentioned, in doses of 0·5 c.c., 1 c.c. and 3 c.c. at intervals of 2, 4, and 6 days in different series of experiments. The blood of the animals was collected after 2, 3, 4, and 5 injections at the time when the next injection would have been due, and the serum separated from the corpuscles by filtering or allowing them to settle. The clear serum was then drawn up into a glass tube, and after it an equal volume of the diluted egg albumen solution, and the end of the tube sealed in the flame. The fluid in the tube remained perfectly clear in every case, and there was no trace of a precipitate either at the junction of the serum and albumen solution, or on shaking. After standing for twelve hours or more in the case of the serum of the *Pectens*, a slight cloud was sometimes produced, but this was always

found to be due to bacterial growth, and in cases where the serum and albumen solution were not shaken together, was always confined to the serum and was not more pronounced at the junction of the liquids. In the case of the serum of the coelomic fluid of the *Echini*, this growth was not apparent till much later, and it was not found at all in the case of the serum of *Arca*. If particular care was taken to collect the serum under aseptic precautions the formation of this cloud due to bacterial growth could be entirely prevented.

A further series of experiments was undertaken on the *Echini* in which very much larger doses of albumen were injected. In one series as much as 50 c.c. of the albumen diluted 1 in 5 was injected at 2-day intervals into the coelomic cavity: this had no ill effect on the animals, but rather seemed to act as a stimulant, locomotion appeared to be more rapid, and the tube feet that were unattached to any neighbouring object were usually fully extended, and their waving motion was more pronounced and more rapid than normal. After large doses such as these there was also no trace of a precipitin action. An examination of the coelomic fluid twelve hours after such an injection, showed no perceptible increase in the albuminous contents as roughly measured by precipitation with Esbach's reagent, hence it would seem that the albumen must be rapidly eliminated from the coelomic fluid.

Larger doses, such as 50 c.c. of egg albumen diluted with an equal volume of sterile sea water, had a toxic action, and caused the death of an *Echinus* of average size in about 24 hours.

As the results of these experiments on six different species of animals were so uniformly negative, it did not seem profitable to continue the work, but as a check on the methods some experiments were performed on the production of a precipitin to egg albumen in fish, species of *Pleuronectes* and *Ruia* being used for the purpose. A well-marked precipitin reaction was obtained with the serum of these animals after two injections of 1 c.c. of the albumen diluted with four volumes of sea water at four day intervals.

The experiments described in this note were performed partly at the Plymouth Laboratory of the Marine Biological Association of the United Kingdom, and partly at the Laboratory of the United States Fishery Commission at Beaufort, North Carolina. My thanks are due to both these Laboratories for extending to me facilities for work.

## THE RELATION OF THE REACTION OF THE CULTURE MEDIUM TO THE PRODUCTION OF HAEMOLYSIN.

BY E. E. ATKIN.

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Director Dr W. Bulloch.*)

1 Chart in text.

EVER since artificial media have been used for the cultivation of micro-organisms, the question as to what is the best reaction—that is the degree of acidity or alkalinity—has been a moot point. It has been assumed gratuitously that there is an optimum reaction which is the same for all the common bacteria met with in the laboratory. In recent years however some attempts have been made to settle the question definitely, notably by Eyre and Deeleman. On enquiring what reaction of medium was adopted in six different London laboratories, Eyre found that very divergent standards were used, ranging from 20 units of acidity to 10 units of alkalinity on his scale. (20 units of acidity implies that 20 c.c. of normal alkali must be added to each litre to make it neutral to phenolphthalein. In the case of alkalinity the indicated number of c.c. of normal acid must be added.) The importance of having a definite reaction had until recently not impressed itself upon the majority of bacteriologists. The possibility that although many organisms may grow on media of comparatively widely differing reactions, yet only one of these reactions may be suitable for the growth of a more delicate kind, had been overlooked. Consequently they failed to grow bacteria which by careful adjusting of the reaction of the medium could be readily cultivated. Martin (1910), for example, has lately pointed out the ease with which gonococcus can be made to grow

on serum agar if the reaction is carefully adjusted. In later years the subject has assumed still more importance in connection with the production of bacterial toxins, which have been submitted to a large amount of investigation. In the production of diphtheria toxin it was soon recognised that bouillon containing more alkali than is usually employed for ordinary bacteriological work, yielded the most toxin, and even various devices were invented to avoid the initial production of acid which usually takes place as the consequence of bacterial growth in a sugar-containing medium. Madsen (1896) has made a thorough examination of the factors governing the production of diphtheria toxin. By following the reaction of a growing culture from day to day he found two different types of growth occur,—one in which the medium increases in acidity without abatement, and such cultures are atoxic; in the other the primary increase in acidity is followed by a decrease, and subsequent increase in alkalinity. These cultures are sometimes toxic. The two conditions seemed to have relation to the amount of alkali added in the preparation of the bouillon, in that a strongly alkaline medium gave an alkaline end reaction while a less alkaline medium gave sometimes an acid and sometimes an alkaline end reaction.

In recent years many different toxins have had to be prepared for experimental purposes and the inconstant amount of toxin produced on different occasions has been a source of trouble to many workers. The following experiments have been undertaken to see what part the reaction of the medium plays in the production of a toxin, whether, for example, there is a sharply marked optimum reaction, and if so where it lies, or whether the production is about the same in any medium in which the bacterium grows. The organism with which I have worked is *Vibrio nasik* from which a considerable yield of haemolysin can be obtained and which is susceptible of fairly exact measurement. At the outset it was necessary to define its limits of growth in media of increasing alkalinity and acidity, and at the same time I have compared it with several other organisms commonly met with.

### I. *Determination of the Limits of Growth.*

The bouillon was made from fresh meat and contained  $1\frac{1}{2}\%$  pepton. After filtration NaOH was added until the reaction was just alkaline to phenolphthalein. It was then autoclaved for 15 minutes at  $115^{\circ}\text{C}$ . and filtered *after cooling*, as more precipitate separates out at a lower temperature. The reaction was now tested and more NaOH added if

necessary to make it slightly alkaline. It was autoclaved again and usually a slight precipitate appeared which was removed. The reaction was then adjusted so as to be exactly neutral to phenolphthalein by adding HCl to neutralise excess of NaOH. Test tubes were filled with 8 c.c. of this bouillon. The required reactions were obtained by adding quantities of normal solutions of NaOH or HCl, as the case might be, to the tubes. The tubes were then sterilised. This method was adopted as being the one most commonly used in laboratories when preparing bouillon. The final reaction of the bouillon to indicators after sterilising was not taken into account, as it is probably not significant when a known amount of alkali or acid has been added. The number and nature of the substances in bouillon acting upon indicators make the estimation of the alkalinity or acidity of various samples of doubtful importance, as we do not know to what substances the total alkalinity or acidity is due. It seems a more rational proceeding to start from a fixed point which can be determined with a fair amount of accuracy—the neutral point to phenolphthalein—and vary the quantities of the single constituents, alkali or acid. If the bouillon is always prepared in the same way it is then easy to get it uniform each time. The tubes containing the greatest quantities of alkali and also of acid showed slight precipitates which were neglected. They partly dissolved at the temperature of incubation, 37°. It is interesting to note that they occurred in both alkaline and acid reactions. The tubes were inoculated with young cultures of the organisms investigated, 24-48 hours old, and placed in an incubator at 37°. They were examined after 24 hours and again after 5 days' incubation. The tubes were examined with the naked eye for turbidity, the microscope being employed in doubtful cases, for example with streptococcus. The whole series of organisms was tested at the *same* time and on the *same* preparation of bouillon, and the different members are therefore comparable. The results are set forth in Table I. The double cross ++, indicates the tubes in which growth was visible in 24 hours. The single cross + marks the supplementary tubes which showed growth after 5 days. The former have been enclosed by a dark line to bring into relief those tubes in which growth took place most easily. The numbers indicate the content in NaOH or HCl at the rate of c.c. of normal solutions to a litre.

It will be noticed that the extent of growth varies considerably with the different bacteria, but there seems to be a common optimum roughly about the neutral point, judging from the results of the first day's growth. This is of importance as it shows that bouillon of one standard reaction

may be used for general laboratory purposes, although in certain cases it may be advisable to modify the reaction somewhat. Deeleman (1897) working by a different method also finds that the optimum of growth does not vary greatly for different bacteria. Starting with the blue-litmus neutral point he added different quantities of alkali to gelatin medium and counted the colonies under the microscope. He found the optimum growth usually occurs when from 0·17 c.c. to 0·34 c.c. %  $\frac{N}{1}$  NaOH is added. This corresponds to about 21 to 23 acidity units on Eyre's scale, and seems to be an unusually acid medium. But the

TABLE I.

	Alkaline								Neutral	Acid					
	80	70	60	50	40	30	20	10		10	20	30	40	50	
<i>Vibrio nasik</i>					+	+	++	++	++						
<i>V. cholerae</i>	+	+++	++	++	++	++	++	++	++	++	++				
<i>B. pyocyanus</i>	+	+	+	++	++	++	++	++	++	++	++				
<i>Staphylococcus aureus</i>	+	++	++	++	++	++	++	++	++	++	++				
<i>B. anthracis</i>									+	++	++				
<i>B. prodigiosus</i>					+	++	++	++	++	++	++				
<i>B. typhosus</i>					+	++	++	++	++	++	++				
<i>Proteus vulgaris</i>						++	++	++	++	++	++				
<i>B. dysenteriae</i> (Flexner)						+	++	++	++	++	++				
<i>B. subtilis</i>						+	++	++	++	++	++				
<i>B. acidi lactici</i>						+	++	++	++	++	++				
<i>B. mesentericus</i>						+	++	++	++	++	++				
<i>M. melitensis</i>						+	++	++	++	++	++				
<i>B. coli communis</i>						+	++	++	++	++	++				
<i>B. diphtheriae</i>										++	++	++			
<i>B. megatherium</i>										+	+	++			
<i>Streptococcus</i> (Joint)										+	+	++			
<i>Streptococcus</i> (Blood)										++	++	++			

The numbers at the heads of the columns represent the number of cubic centimetres of  $\frac{N}{1}$  NaOH, or  $\frac{N}{1}$  HCl as the case may be, which have been added to a litre of bouillon neutral to phenolphthalein.

The double cross represents visible growth in 24 hours. The single cross marks the supplementary tubes in which visible growth took place after five days.

neutral point to litmus is not determinable with anything like the degree of accuracy that the neutral point to phenolphthalein is and two readings may differ widely.

TABLE II.

Day of Incubation	Quantities giving equal haemolysis				
	Alkaline		Neutral	Acid	
	15 c.c.	5 c.c.	0 c.c.	5 c.c.	10 c.c.
4	0·022	0·02	0·02	0·04	0·065
5	0·017	0·017	0·017	0·022	0·04
6	0·017	0·017	0·025	0·017	0·017
7	0·017	0·02	0·04	0·017	0·017
9	0·13	0·08	Trace in 2·0	0·017	0·025
10	2·0	Nil	—	0·025	0·04
11	—	—	—	0·04	0·13
12	—	—	—	0·17	0·8
13	—	—	—	0·65	0·8
16	—	—	—	0·5	Nil

In my experiments in almost every case the limit of growth was extended after the first day and in the majority this took place at the alkaline end. This may be taken to indicate that a degree of alkalinity which partially inhibits growth can be overcome if not too excessive. This may be brought about by a slight growth forming a small amount of acid from the sugars present which neutralises some of the alkali and so brings the reaction of the medium gradually within the limits of vigorous growth, more rapid multiplication then taking place. This view is confirmed by an incident I have met with in the case of *Vibrio nasik*. Having found its alkaline and acid limits of growth in the above manner, I have inoculated flasks of bouillon of 300–500 c.c. with reactions covering this range and have invariably found that the flasks close to the alkaline limit, as indicated in test tubes of 8 c.c. fluid, did not grow, the reason presumably being that the larger amount of alkali was too great for the bacteria to overcome in the manner suggested.

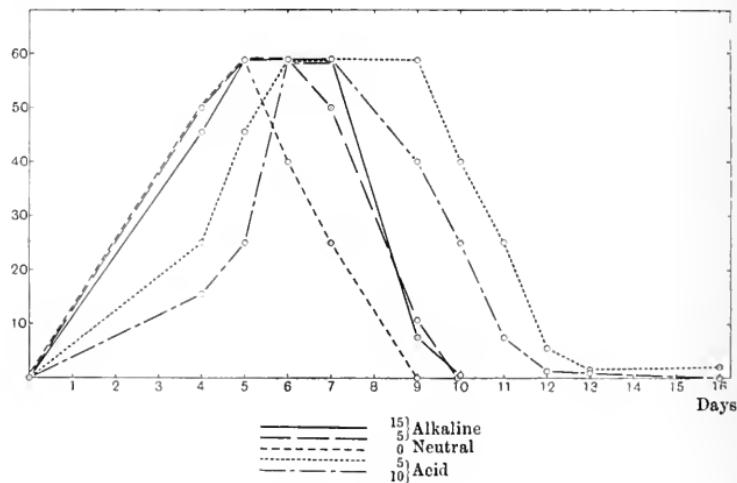
## II. Determination of the Optimum Reaction for Haemolysin Production.

The haemolysin was that produced by *Vibrio nasik*. Five litre flasks of bouillon containing 275 c.c. of varying reactions were inoculated with young cultures, and small samples were removed from time to time and shaken with toluol. They were all kept in an ice safe until the end of the experiment and all tested on the same blood on the same day.

employing the technique instituted by Madsen. In estimating the haemolysin in a given sample, varying amounts of the fluid were put into test tubes and made up to 2 c.c. with 0·9% NaCl solution, and then 8 c.c. of a 1% suspension of washed sheep red corpuscles forcibly introduced with a syringe to ensure immediate mixing. The tubes were then shaken, heated for  $2\frac{1}{2}$  hours at 37°, again shaken and placed in an ice safe until next day. One tube showing about 30–40% haemolysis was chosen and matched with others of similar tint throughout the different series. The reciprocals of the quantities giving equal haemolysis were then charted.

From an examination of the chart several points of interest are discovered.

#### Haemolysin



Haemolysin production of *Vibrio nasik*. Bouillon of different reactions.

The production of haemolysin in the case of *Vibrio nasik* is practically co-extensive with its range of growth in media of varying reactions. It is probably a function of the organism's vigorous growth and is only absent in cultures when this is partially inhibited, that is in the most alkaline and most acid media in which multiplication is just possible. Moreover it will be seen that each curve has attained the same height although at different times and lasting for different periods of time. Thus the power of producing haemolysin is not altered at all

by changing the reaction of the bouillon; its appearance and disappearance only are modified.

The production of haemolysin in the alkaline and neutral bouillon cultures took place distinctly earlier than in the acid ones. It seems reasonable to suppose that this is due to the excess of alkali quickly neutralising the acid formed from the decomposition of sugars, whereas in acid media the organisms have themselves to form the necessary alkali from the pepton. Bacterial growths in bouillon if left long enough nearly always become alkaline from decomposition of the pepton, and anything which favours this process would be likely to encourage the speedier appearance of lysins which are derivatives of the nitrogenous bodies. We know that proteids are attacked sooner in sugar-free media, as indol is produced earlier in such a case.

When the amount of haemolysin has attained its greatest height it remains stationary for a short period. A curious flat-topped curve is thus produced. Walbum has found that this is dependent upon an insufficiency of pepton at the point where the flat top begins. His hypothesis is that the bacteria produce a prolysin not haemolytic in itself, but by further action upon pepton giving rise to true haemolysin. This view is supported by the fact that if a sample is taken from any point between the two bounding points of the flat top and pepton added, an increase in the amount of haemolysin always takes place. Upon what the duration of this flat top depends in different experiments it is difficult to say. I believe however that it has some relation to the volume of the culture fluid, for when using larger amounts of bouillon (500 c.c.) than in the present instance I have noticed that the condition lasts longer. In the present experiment all the curves show a flat top except the "neutral bouillon" one. This curve has a fastigium of such short duration that if daily samples had not been taken it would have been entirely missed, and it shows on the chart as a point. The descending limbs of the curves can be well compared in this case as they fall from the same altitude. The point to notice is that the curve representing the most alkaline bouillon has the steepest gradient probably due to the destroying action of the excess of alkali present. Famulener and Madsen (1908) have investigated the increase in the rate of destruction of vibriolysin by heat, caused by alkalis and acids. To vibriolysin, the reaction of which corresponded to one acid unit on Eyre's scale, they added increasing amounts of NaOH and HCl, and compared their rates of destruction at a fixed temperature. The alkaline samples quickly showed an increased rate; and the acid only after a considerable

amount had been added well outside the limit of growth of *nasik* in acid media. Then however the rapidity of increase was greater than in the case of alkali. My experiments are in unison with this result, in that the most alkaline bouillon shows an increased rate of destruction whereas the most acid bouillon does not. It would seem from an inspection of the curves that the acid culture bearing the number five holds its haemolysin for the longest period of time, which would imply that at this point it is least affected by the alkaline or acid content. Thus although there is no optimum of quantity as regards the production of haemolysin in bouillon of different reactions, yet there is an optimum of duration of haemolysin which seems to be about the point where the bouillon requires 5 c.c. of normal NaOH per litre to neutralise it to phenolphthalein.

This work was carried out under a grant from the London Hospital Research Fund.

I am indebted to Dr William Bulloch for much kind assistance during the course of this research.

#### CONCLUSIONS.

1. Many bacteria have an extensive range of growth in bouillon of varying reactions; some have a much more restricted range, as the streptococci obtained from the blood stream.

2. There seems to be roughly a common optimum of growth when the reaction of the bouillon is neutral to phenolphthalein, provided that the medium is always prepared in the same way as described above. Although small differences may be desirable for individual bacteria, this reaction can be recommended for ordinary laboratory routine work.

3. Most micro-organisms have some power of overcoming the inhibitory effect of excess of NaOH. This is much commoner than the capacity for overcoming excess of HCl which was observed in a few instances.

4. The haemolysin production of *Vibrio nasik* is not limited to an optimum reaction, but is practically co-extensive with its range of growth in media of varying reactions. In a series of bouillon cultures containing different quantities of alkali and acid, the amount of haemolysin produced was about the same in each case, but the *time* at which the maximum was reached was influenced by the reaction. This was modified

in such a way that the most alkaline cultures produced haemolysin soonest and lost it again before the most acid ones. There seemed to be an optimum as regards *length of duration* of haemolysin in the culture in the case of the sample bearing the reaction, acid 5.

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## ATTEMPTS TO REPRODUCE THE TYPHOID-CARRIER STATE IN THE RABBIT.

(PRELIMINARY COMMUNICATION.)

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With Plate III.

SINCE the work of Blachstein and Welch (1891) who showed that rabbits inoculated intravenously with the typhoid bacillus might continue to harbour that organism in the gall-bladder for a prolonged period, much help in the elucidation of the typhoid-carrier state in man has come from the results of experimental investigation of this condition in the rabbit.

Among those who have contributed most materially to our knowledge of this subject one may cite the names of Forster and Kayser (1905), Doerr (1905) and Koch J. and Chiarolanza (1909), the latter of whom has shown that the infection of the gall-bladder following intravenous inoculation of *B. typhosus* in the rabbit most probably takes place directly through the blood vessels of the gall-bladder wall, although a secondary invasion by infected bile from the liver may certainly take place at a somewhat later period.

Histological examination of the gall-bladder and biliary tracts in such acute cases has revealed the presence of bacillary dépôts of embolic origin at the extremities of the rugose papillary formations which give the mucosa the shaggy appearance so characteristic of typhoidal infection in the human gall-bladder.

In rabbits which have been killed at a later period after inoculation and found to be still harbouring the typhoid bacillus, very similar changes have been noted. Thus in one of Chiarolanza's experimental

rabbits which was sacrificed on the 14th day after inoculation, the gall-bladder mucosa showed inflammatory foci near the tips of the shaggy papillae, but bacilli could not always be demonstrated in them by staining methods, although a pure culture of the *B. typhosus* was obtained.

It is obvious that this method of experimentation offers a wide prospect of studying the carrier-state under laboratory conditions and particularly with a view to treatment. Already some preliminary experiments by Conradi (1910) and Hailer and Rimpau (1911) have appeared in which some success has been achieved in freeing the animal from typhoid bacilli by the intrarectal injection of Chloroform, Bromoform, Iodoform and other substances.

Most of these therapeutic attempts have been begun as a rule not later than the first fortnight after infection but a small series of animals was experimented upon by Conradi about the 25th day and in these cases recourse was had to preliminary laparotomy in order to determine whether the gall-bladders were really infected at this date.

The experiments which I propose to record in this short note were commenced in Oct. 1910 and the main object in view was to determine the frequency with which the chronic carrier condition occurs in the rabbit after intravenous inoculation and the length of its duration. It was also hoped that a clue to the infected condition of the gall-bladder might be obtained from the systematic bacteriological examination of the faeces. If such examination proved successful it was intended also to institute various therapeutic measures with a view to influencing the gall-bladder lesions.

#### *Author's Experiments.*

Between the dates Oct. 17, 1910, and Oct. 29, 1910, twelve rabbits with an average weight of 2·52 kilos received a single intravenous inoculation of 4 c.c. of a 24-hour broth culture of *B. typhosus*. In six of the animals a strain isolated from an intestinal carrier was employed, and in the other six a strain from a urinary carrier.

Two animals died within 12 hours from the effects of the injection.

The systematic examination of the faeces of the remaining ten rabbits was begun about ten days after inoculation. Before plating on MacConkey's medium the faeces were incubated for 24 hours at 37° C. in 0·5% malachite-green broth.

The agglutinin-content of the rabbits' sera was also estimated at intervals, the homologous organism being employed.

*Results in the individual rabbits.*

*Rabbit I.* Wt. 2·50 kilos. Inoculated on Oct. 17, 1910.

Agglutinins: 31 Oct., 1 in 2000 + + + ; 9 Nov., 1 in 2000 + + + ; 15 Nov., 1 in 2000 + + ; 27 Nov., 1 in 2000 + + ; 13 Jan. 1911, 1 in 2000 + + + ; 21 Feb., 1 in 2000 + .

Faeces: Exams. on 1 Nov., 9 Nov., 17 Nov., 23 Nov., 1 Dec., 12 Jan., 4 Feb., 21 Feb., 1 Mar., 3 Mar. All negative.

*Rabbit IV.* Wt. 1·85 kilos. Inoc. Oct. 18, 1910.

Agglutinins: 31 Oct., 1 in 2000 + + + ; 9 Nov., 1 in 2000 + + + ; 15 Nov., 1 in 2000 + + + ; 29 Nov., 1 in 2000 + + ; 13 Jan., 1 in 2000 + + + ; 21 Feb., 1 in 2000 + + .

Faeces: Exams. on 1 Nov., 9 Nov., 17 Nov., 23 Nov., 1 Dec., 12 Jan., 4 Feb., 21 Feb. All negative.

*Rabbit V.* Wt. 3·50 kilos. Inoc. 21 Oct. 1910.

Agglutinins: 31 Oct., 1 in 2000 + + + ; 9 Nov., 1 in 2000 + + + ; 22 Nov., 1 in 2000 + + ; 29 Nov., 1 in 2000 + + ; 13 Jan., 1 in 2000 + + + ; 21 Feb., 1 in 500 + .

Faeces: Exams. on 1 Nov., 14 Nov., 17 Nov., 23 Nov., 1 Dec., 12 Jan., 4 Feb., 21 Feb. All negative.

*Rabbit VI.* Wt. 2·68 kilos. Inoc. Oct. 22, 1910.

Agglutinins: 31 Oct., 1 in 2000 + + + ; 9 Nov., 1 in 2000 + + + ; 22 Nov., 1 in 2000 + + + ; 29 Nov., 1 in 2000 + + + ; 13 Jan., 1 in 2000 + + + ; 21 Feb., 1 in 2000 + + .

Faeces: Exams. on 1 Nov., 14 Nov., 17 Nov., 23 Nov., 1 Dec., 12 Jan., 4 Feb., 21 Feb. All negative.

*Rabbit VII.* Wt. 2·0 kilos. Inoc. Oct. 24, 1910.

Agglutinins: 1 Nov., 1 in 2000 + + + ; 10 Nov., 1 in 2000 + + + ; 22 Nov. 1 in 2000 + + + ; 30 Nov., 1 in 2000 + + + .

Faeces: **Positive** on 4 Nov. 1910.

Further examinations on 14 Nov., 19 Nov., 28 Nov., 1 Dec. were all negative.

Animal died on 6 Jan. 1911 (see below).

*Rabbit VIII.* Wt. 2·65 kilos. Inoc. Oct. 25, 1910.

Agglutinins: 1 Nov., 1 in 2000 + + + ; 10 Nov., 1 in 2000 + + + ; 22 Nov., 1 in 2000 + + + ; 30 Nov., 1 in 2000 + + + ; 24 Jan., 1 in 500 + + , 1 in 2000 O; 23 Feb., 1 in 500 + + .

Faeces: Exams. on 4 Nov., 14 Nov., 19 Nov., 28 Nov., 1 Dec., 18 Jan., 6 Feb., 22 Feb. All negative.

*Rabbit IX.* Wt. 2·35 kilos. Inoc. Oct. 26, 1910.

Agglutinins: 1 Nov., 1 in 2000 + + + ; 10 Nov., do.; 28 Nov., do.; 6 Dec., do.; 24 Jan., 1 in 2000 + ; 23 Feb., 1 in 2000 + .

Faeces: Exams. on 4 Nov., 15 Nov., 19 Nov., 28 Nov., 5 Dec., 18 Jan., 6 Feb., 22 Feb. All negative.

Animal showed paralysis of hind legs and diarrhoea and was killed (see below).

*Rabbit X.* Wt. 2.85 kilos. Inoc. Oct. 27, 1910.

Agglutinins: 7 Nov., 1 in 2000++ ; 10 Nov., 1 in 2000++ ; 28 Nov., 1 in 2000++ ; 6 Dec., do.; 24 Jan., 1 in 2000+.

Faeces: **Positive** on 4 Nov. 1910.

Further examinations on 15 Nov., 19 Nov., 28 Nov., 5 Dec., 18 Jan., 6 Feb. were all negative.

Animal died on 13 Feb. 1911 (see below).

*Rabbit XI.* Wt. 1.97 kilos. Inoc. Oct. 28, 1910.

Agglutinins: 1 Nov., 1 in 2000++ ; 15 Nov., do.; 28 Nov., do.; 6 Dec., do.; 24 Jan., 1 in 2000+ ; 23 Feb., 1 in 500++ , 1 in 2000 O.

Faeces: Exams. on 9 Nov., 15 Nov., 23 Nov., 28 Nov., 5 Dec., 6 Feb., 22 Feb. All negative.

*Rabbit XII.* Wt. 2.17 kilos. Inoc. Oct. 29, 1910.

Agglutinins: 7 Nov., 1 in 2000+ ; 15 Nov., 1 in 2000++ ; 28 Nov., do.; 6 Dec., do.; 25 Jan., do.; 23 Feb., 1 in 2000+.

Faeces: Exams. on 9 Nov., 15 Nov., 23 Nov., 28 Nov., 5 Dec., 13 Feb., 22 Feb. All negative.

In two instances only, therefore, was the typhoid bacillus recovered from the faeces, at the end of eight days after inoculation in the case of Rabbit X and at the end of 11 days in the case of Rabbit VII. Rabbit VII was found dead on 6 Jan. 1911, i.e. about  $2\frac{1}{2}$  months after inoculation. The *B. typhosus* was recovered in pure culture from the bile and gall-bladder wall but not from the heart blood or colon. Plate III shows a cross section of the gall-bladder in this case. Apart from the gall-bladder lesion and the finding of *B. typhosus* no obvious cause of death could be found. Although the heart blood gave a negative result, the possibility is not excluded that death may have been due to an autoinfection. One has also to consider the possibility of some anaphylactic disturbance as a contributory cause of death owing perhaps to the sudden liberation of typhoid antigen from the gall-bladder area. Dr Ledingham informs me that on several occasions he has isolated *B. typhosus* from the gall-bladder of guinea-pigs which had received sublethal doses of the living organism 4-5 weeks previously.

*Rabbit X* was found dead on 13 Feb., 1911. There were no obvious lesions at autopsy and the *B. typhosus* was not recovered. (Examination of Bile, Gall-bladder wall, Duodenum, Ileum, Colon, Spleen, Liver and Heart Blood.)

*Rabbit IX* was killed after showing paralysis of the hind legs and diarrhoea. *B. typhosus* was not recovered from any of the organs.

*Attempts to exhibit B. typhosus in the faeces by the use of purgatives.*

Owing to the persistently negative results obtained by examination of the faeces in the majority of the rabbits, it was thought that the administration of purgatives might help matters. The rabbits however proved to be extraordinarily resistant to nearly all the well-known purgatives.

The following drings were given by the mouth, one after the other without effect : Mag. Sulph.  $\frac{1}{2}$  oz., Phenolphthalein gr. XV, Pulv. Jalap. Co.  $\frac{1}{2}$  oz., Paraffinum liquidum  $1\frac{1}{2}$  oz., and finally Croton oil in doses of 13 minims.

Arecolin  $\frac{1}{10}$  grain was given hypodermically with no better result, also  $\frac{1}{100}$  grain of Eserine, the only effect of which was to produce violent spasms in the hind legs, lasting for about two hours but which led to no purgation. At last it was found that Calomel in doses of 4 grains produced slight purgation on the following day but not invariably. A dose of Calomel was therefore given to each rabbit on the day before the faeces was collected but in spite of the purgation it was impossible to recover the typhoid bacillus.

*Laparotomy and Puncture of Gall-bladder.*

Between Feb. 28, 1911, and Mar. 9, 1911, laparotomy was performed on the remaining seven rabbits but in four only was the gall-bladder found infected at the operation. In all four cases the bile, removed by puncture, proved sterile. One cannot conclude, however, that the animals are uninfected as it has been shown that the bile may be free from typhoid bacilli while the bladder wall may still yield a positive result on culture.

All the animals made an excellent recovery from the operation and it is proposed to reinoculate them in order to determine whether, according to Fornet's view (see Ledingham, 1910), the carrier-state is more likely to follow a second infection.

The blood of these 7 rabbits was still found to agglutinate the typhoid bacillus, four months after injection in a dilution of 1 in 500.

**SUMMARY.**

Preliminary record of attempts to produce the typhoid-carrier state in the rabbit and to obtain evidence of the presence of this condition by bacteriological examination of the faeces.





Illustrating the paper by Mr H. de R. Morgan.  
(Description: see p. 207.)

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## DESCRIPTION OF PLATE III.

Section of mucosa of gall-bladder (Rabbit VII), from which *B. typhosus* was recovered at death 2½ months after inoculation. Rugose condition well marked. Inflammatory foci with numerous chromatic debris (A, B, C) present in some of the papillae, and in one of them (A) the focus surrounds the central vessel of the papilla. Organisms were not definitely recognised in these foci by staining methods. These partially necrotic foci obviously represent the advanced stage of the embolic bacillary dépôts observed in early cases.

ON THE ALTERATIONS IN HAEMOLYTIC IMMUNE-BODY WHICH OCCUR DURING THE PROCESS OF IMMUNISATION<sup>1</sup>.

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DURING the process of immunisation with formed elements, *e.g.* red blood corpuscles, the amount of immune-body in the serum of the immune animal may undergo an enormous increase. Thus on injecting a rabbit with ox blood, its serum from possessing practically no immune-body may become so active that 0'00025 c.c. suffices to cause complete lysis of 1 c.c. of 5 per cent. blood suspension in the presence of sufficient complement. The estimation of the amount of immune-body necessary to effect lysis of a given quantity of blood suspension at different periods obviously gives no information as to whether or not the molecules of immune-body undergo in addition a qualitative change in the course of immunisation. As Muir<sup>2</sup> says, "the mere occurrence of haemolysis gives no indication of the total combining affinities" and he has always insisted on the importance of studying the combination of the components concerned in haemolysis by the absorption method. Accordingly we have investigated by this method the changes occurring in the molecules of immune-body during the process of immunisation.

<sup>1</sup> We have much pleasure in acknowledging our indebtedness to the Carnegie Trust for a grant toward the expenses of this research.

<sup>2</sup> *Studies on Immunity*, Oxford, 1909.

For this purpose we have chosen the immune-body obtained by injecting washed ox-blood into the peritoneal cavity of rabbits, since in this way a very active immune-body is usually developed, which along with guinea-pig's complement has an extremely powerful toxic effect as judged by the small quantities of both components required to cause haemolysis. Further, the combining properties of such immune-bodies, when raised to a high degree of potency, have already been thoroughly investigated by Muir. Thus, in general, the immune sera studied by Muir were obtained by giving three intraperitoneal injections of the corpuscles of 5, 10 and 15 c.c. of ox-blood at intervals of ten days and then bleeding 7 to 10 days after the last injection. The sera were inactivated by heating at 55° C. Such an immune-body was found to have the following properties:

(1) To the suspension of blood corpuscles from six to ten times the minimum haemolytic dose of immune-body requires to be added before one dose remains free, sufficient time being allowed to permit of the maximum degree of combination.

(2) When the immune-body and complement are added to blood corpuscles there occurs in addition to haemolysis an absorption of the complement. The combined complement does not become dissociated again to any appreciable extent.

(3) The amount of complement absorbed may be much greater than the amount necessary to cause just complete lysis, and the degree of complement fixation depends on the amount of immune-body present. Thus, multiple doses of immune-body together with red corpuscles lead to the union of increased amounts of complement, and in some instances the complement taken up is approximately proportional to the amount of immune-body; in other cases considerable divergence is met with, mainly in the direction of diminished proportional absorption in the case of increasing multiples of immune-body.

Our observations have reference to the points mentioned above. The sera of seven animals have been examined, each being repeatedly tested at different stages of immunisation. The sera were allowed to separate from the blood-clot for 24 hours and were sealed in quill tubes and then heated once for 1½ hours at 55° C. They were then kept in the dark at room temperature. Tests were made with the same specimen of serum on repeated occasions, so that its properties were usually estimated both recently after withdrawal from the body and also after the lapse of from several weeks to several months. By way of correlating the results several specimens of serum from the

same animal and also from different animals were usually examined simultaneously; and an immune serum of high potency was included in the series. In this way additional information was obtained as to the part played by individual properties of different specimens of red blood corpuscles, especially as regards the effect of age on the immune serum. It will be best to refer to the latter points first of all, since they afford an indication as to the interpretation which should be put upon varying results in consecutive experiments.

#### *Methods.*

The procedure followed in the experiments was that adopted by Muir. In order to obtain blood suspensions of fairly uniform density the mixture of washed blood corpuscles and salt solution was centrifugalised until there was a sharp line of demarcation between sediment and supernatant fluid; the latter was then pipetted off and 3 c.c. of sediment were made up to 100 c.c. with salt solution. The guinea-pig's complement was freed from natural immune-body by treatment with an equal volume of washed ox-blood for an hour at 0° C. The haemolytic doses of the immune sera were always estimated just before the experiment and with the same complement and corpuscles; when necessary, to ensure accuracy of measurement, the sera were diluted 10 times or 100 times with salt solution immediately before the estimation. The amount of immune-body necessary to produce just complete lysis in 1½ hours at 37° C. in the presence of 5-10 doses of complement was taken as the dose.

#### *The dosage of immune-body as affected by individual properties of the red blood corpuscles.*

*Example.* Three specimens of ox-blood were obtained—No. (1) on 6. vi. '10, Nos. (2) and (3) on 7. vi. '10. The blood was kept on ice till 8. vi. '10, when suspensions of the washed corpuscles were prepared and the dose of a potent immune serum tested. Each tube containing 1 c.c. of suspension along with immune-body (I.B.) received 0·05 c.c. of guinea-pig's complement (7 doses) and the reading was taken after 2½ hours at 37° C. The results were as follows:—

R.C.'s No. (1)	dose of I.B.	= 0·0007 c.c.
" "	(2) "	= 0·0005 "
" "	(3) "	= 0·0003 "

These results show that in the presence of excess of complement the "sensitising action" of a given immune-body varies greatly with different specimens of blood. Such variations in susceptibility must explain in great part the erratic alterations in dosage of the same immune serum met with when tested at different times (a different specimen of ox-blood and complement was of course employed on each occasion).

*Examples.* (1) Immune serum from rabbit "B" withdrawn 22. v. '10, four days after the second injection. Table I shows the haemolytic dose in a series of estimations of this immune serum with different samples of blood.

TABLE I.

Blood	Date of estimation	Dose for 1 c.c. R.C.'s
a	25. v. '10	0·003 c.c.
b	1. vi. ,,	0·006 ,,
c	23. vi. ,,	0·002 ,,
d	9. vii. ,,	0·0045 ,,
e	13. viii. ,,	0·0035 ,,
f	3. viii. ,,	0·0045 ,,
g	14. xii. ,,	0·006 ,,

(2) The following example is of interest as the immune-body was exceedingly powerful. Immune serum withdrawn viii. '10, ten days after the third injection.

TABLE II.

Blood	Date of estimation	Dose for 1 c.c. R.C.'s
a'	3. viii. '10	0·0005 c.c.
b'	24. ix. ,,	0·0006 ,,
c'	11. xl. ,,	0·0008 ,,
d'	14. xii. ,,	0·00075 ,,
e'	23. xii. ,,	0·0012 ,,
f'	22. ii. '11	0·0014 ,,
g'	1. iii. ,,	0·0012 ,,

The immune serum in this case appeared to be diminishing in potency. The results of tests which were carried out simultaneously to determine the amount of complement absorbed by red corpuscles along with the immune-body (one and five doses) are given in Table III.

The method of such an absorption experiment is as follows:—To a series of test tubes containing 0·5 c.c. of 5 per cent. blood suspension and a given number of doses of immune-body (usually one and five doses)

varying amounts of complement are added. The tubes are incubated for  $1\frac{1}{2}$  hours at  $37^{\circ}\text{C}$ . and are shaken at intervals during this time. Then if lysis is incomplete in any tubes, these are centrifugalised and the fluid is pipetted off into other tubes. To each tube of clear fluid 0.5 c.c. of blood suspension, sensitised previously with five doses of a powerful immune serum from the rabbit, is added. The tubes are again incubated for  $1\frac{1}{2}$  hours and kept at room temperature overnight; the result is read next morning.

TABLE III.

Blood	Amount of complement required to cause just complete lysis of the test corpuscles	
	1 dose of I.B.	5 doses of I.B.
a' (3. viii. '10)	0.035 c.c.	0.18 c.c.
c' (11. xi. "	0.03 "	0.2 "
d' (14. xii. "	0.02 "	0.09 "
e' (23. xii. "	0.045 "	0.15 "
f' (22. ii. '11)	0.03 "	0.12 "
g' (1. iii. "	0.045 "	0.13 "

When allowance is made for the variations due to different specimens of red corpuscles and complement, it will be seen that the immune-body has not undergone any definite alteration so far as its power of causing combination of complement with the red corpuscles is concerned. Accordingly, these results confirm the view that the immune-body is fairly stable in this respect, and it is clear that if it does deteriorate with age the alteration does not markedly affect the relative amounts of complement absorbed with one dose and with multiple doses. That is to say, deterioration with age might simply be due to a disappearance of a proportion of the molecules without any qualitative alteration.

#### *Alterations in the immune-body during the process of immunisation.*

The following examples illustrate the general principle which we have found to hold in all cases. The experiments were all carried out in the manner described above; 0.5 c.c. of blood suspension and immune-body (one or five doses) was mixed with complement; after  $1\frac{1}{2}$  hours at  $37^{\circ}\text{C}$ . the presence of free complement in the fluid was tested for by adding corpuscles sensitised with five doses of a powerful mature immune serum (in some cases 0.5 c.c. of test corpuscles was employed, in other experiments 1 c.c., as is noted in the tables).

By "mature" immune-body is meant one which was obtained after repeated injections (usually three), and whose dose was low, 0·001 c.c. or less for 1 c.c. of blood suspension.

Date of experiment, 8. III. '10.

Rabbit "BW," I.B. 28. I., 8 days after 1st injection (4 c.c. blood-sediment),

				D. = 0·05 c.c.
„	4. II. '10, 7	„	2nd „	D. = 0·03 c.c.
„	8. II. '10, 11	„	„	D. = 0·003 c.c.
„	1. III. '10, 7	„	3rd „	D. = 0·008 c.c.
D. of "mature" I.B. = 0·0003 c.c.				

Lysis of test corpuscles (1 c.c. + 5 D. of I.B.)

Amount of complement in c.c. ....	1 dose of I.B.				5 doses of I.B.			
	0·01	0·015	0·02	0·025	0·01	0·02	0·035	0·055
"BW" 28. I.	just complete	complete	complete	complete	complete	complete	complete	complete
„ 4. II.	just complete	complete	complete	complete	just complete	complete	complete	complete
„ 8. II.	almost complete	just complete	complete	0	trace	almost complete	complete	
„ 1. III.	just complete	complete	complete	0	0	trace	almost complete	
Mature I.B.	complete	complete	complete	0	trace	almost complete	complete	

Initial lysis of corpuscles in series with 1 D. of I.B. after 1 hour:

"BW" 28. I., 4th tube just complete.

„ 4. II., 4th „ „

„ 8. II., 1st „ „

„ 1. III., 3rd „ „

Mature I.B. 4th „ „

The result of this experiment shows that, (1) the immune-body in the early stages of immunisation (28. I. and 4. II.) is deficient in the power of leading to the combination of complement. Thus, with multiple doses of such an immune-body, little or no more complement may be absorbed than with one dose (in some other instances multiple doses did cause a slight increase in the complement absorption). As immunisation proceeds (8. II.) the power of absorbing complement is acquired, and it persists well marked after the potency of the serum, as measured by the haemolytic dose, has begun to fall (1. III.); (2) the amount of complement necessary to produce lysis in the presence of a small amount of immune-body varies; but in this respect the specimens of the dates 28. I., 4. II., 1. III. and the mature immune-body are practically comparable, although they show such great differences in the amounts of complement absorbed by multiple doses. The

mature immune-body was older than any of the specimens of immune-body "BW." The interval elapsing between the experiment and the withdrawal of specimen I. III. is comparatively short as compared with the age of the other specimens. The possibility of this factor of age introducing a disturbing effect is eliminated in the following example.

A. Date of experiment, 24. i. '10.

Rabbit "S," I.B. 24. i. '10, 4 days after 1st injection (4 c.c. blood-sediment), D. = 0.0025 c.c. Dose of mature I.B. = 0.00025 c.c.

Amount of complement in c.c. ....	Lysis of test corpuscles (1 c.c.+5 D. of I.B.)				
	0.0125	0.0175	0.025	0.035	0.045
" S " 24. i.	trace	almost complete	just complete	complete	complete
Mature I.B.	trace	trace	marked	very marked	almost complete

B. Date of experiment, 8. iii. '10.

Rabbit "S," I.B. 28. i. '10, 8 days after 1st injection, D. = 0.0012 c.c. D. of mature I.B. = 0.0003 c.c.

Amount of complement in c.c. ....	Lysis of test corpuscles (1 c.c.+5 D. of I.B.)						
	1 D. of I.B.	2 D. of I.B.	3 D. of I.B.	4 D. of I.B.	5 D. of I.B.	6 D. of I.B.	7 D. of I.B.
" S " 28. i.	0.01	0.015	0.02	0.025	0.01	0.02	0.035
" S " 28. i.	almost complete	complete	complete	0	0	trace	almost complete
	complete						complete
Mature I.B.	complete	complete	complete	complete	0	trace	almost complete
							complete

C. Date of experiment, I. iv. '10.

Rabbit "S," I.B. 24. i. '10, D. = 0.01 c.c.

28. i. '10, D. = 0.001 c.c.

Amount of complement in c.c. ....	Lysis of test corpuscles (1 c.c.+5 D. of I.B.)						
	1 D. of I.B.	2 D. of I.B.	3 D. of I.B.	4 D. of I.B.	5 D. of I.B.	6 D. of I.B.	7 D. of I.B.
" S " 24. i.	0.01	0.02	0.03	0.02	0.035	0.05	0.07
" S " 24. i.	very marked	complete	complete	marked	complete	complete	complete
" S " 28. i.	very marked	complete	complete	0	0	very marked	complete

The results show clearly the deficient power of absorbing complement exerted by the immune-body at the early period of immunisation. This is apparent both when the immune serum 24. i. is tested immediately after withdrawal and also after two months.

The possibility that the degree of complement absorption may be influenced by elements of the serum other than the immune-body had to be considered. Accordingly, the following experiments were carried out, (1) an immune serum was tested at various periods of immunisation,

the time of withdrawal of the specimens being so chosen that a specimen taken while the immune-body content was rising after the first injection, had almost the same haemolytic dose as one taken when the immune-body content was falling, and (2) a potent immune serum was diluted with heated normal rabbit's serum until the volume of serum containing a dose was the same as that of a weak rising immune-serum. The complement absorbing powers of the latter and of the diluted and undiluted potent immune serum were then tested simultaneously.

(1) *Experiment illustrating the complement absorption by rising and falling immune-bodies of approximately equal dosage.*

Date of experiment, 3. viii. '10.

Rabbit "B," I.B. 9. v. '10, rising, 4 days after 1st injection (4 c.c. blood-sediment),  
D. = 0·025 c.e.

„ 22. v. '10, „	4	„	2nd	„	D. = 0·00225 c.e.
„ 7. viii. '10, falling, 26	„	3rd	„	D. = 0·006 c.e.	
„ 2.viii.'10, „	52	„	3rd	„	D. = 0·028 c.e.

D. of mature I.B. = 0·00025 c.e.

Lysis of test corpuscles (1 c.c. + 5 D. of I.B.)

Amount of guinea-pig's complement in c.c. ....	1 D. of I.B.				5 D. of I.B.				
	0·015	0·025	0·035	0·05	0·05	0·07	0·09	0·12	0·15
9. v.	0	0·275	0·6	0·8	0·7	0·875	0·9	complete	complete
22. v.	0	0·4	0·825	complete	0	0·25	0·8	complete	complete
7. viii.	0	0·25	0·5	0·825	0	0	0	0·25	0·7
2. viii.	0	0	0	0	0	0	0	0	0
Mature I.B.	0	0	0·5	0·875	0	0	0	0·15	0·4

Initial lysis in 1 D. series:

"B," 9. v., 4th tube just complete.
22. v., 1st „ „
7. viii., 3rd „ „
2. viii., 2nd „ „
Mature I.B. 3rd „ „

Thus, the specimens of immune serum 9. v. and 2. viii. are almost identical so far as the haemolytic dose is concerned; but the latter leads to the absorption of much more complement. Also (1) the rate of lysis is greater with the falling immune-body and (2) the amount of complement necessary to produce complete lysis in the presence of a limited amount of immune-body (one dose) is less with the falling than with the rising immune-body. As will be seen later, however, there is no definite relationship between the amount of complement necessary to cause complete lysis and the amount of complement absorbed through the agency of a given immune-body.

(2) *Experiment illustrating the effect of diluting the immune-body with inactive serum.*

Date of experiment, 1. iii. '11.

Rabbit "GW," I.B. 28. ii. '11, 12 days after 3rd injection, D. = 0.0022 c.c.

" "BH," I.B. 28. ii. '11, 15 " 1st " D. = 0.006 c.c.

With the I.B. of rabbit "GW" two series of tests were made:

(a) Without any addition:

(b) With the addition of normal rabbit's serum (55° C.), so that 1 D. was contained in 0.006 c.c. of serum.

Lysis of test corpuscles (0.5 c.c. + 5 D. of I.B.)

Amount of guinea-pig's complement in c.c. ....	1 D. of I.B.					5 D. of I.B.				
	0.02	0.03	0.04	0.05	0.04	0.055	0.075	0.11	0.15	
"GW" (a) without serum	0.2	0.25	0.3	0.35	0.55	0	0.1	0.25	0.3	0.6
"GW" (b) with serum ...	0.2	0.25	0.3	0.35	0.55	0	0.1	0.25	0.3	0.65
"BH" ...	... 0.25	0.5	0.75	0.9	com- plete	0	0.25	0.5	com- plete	com- plete

Initial lysis in 1 D. series:

"GW" (a) without serum, 2nd tube just complete.

" " (b) with " 2nd " "

"BH" 2nd " "

Thus, the addition of serum to the potent immune-body ("GW") has caused practically no alteration in its power of absorbing complement along with the red corpuscles. Further evidence that the amount of complement absorption is due to the character of the immune-body molecules and is independent of other serum constituents is furnished by the fact that after a single injection of blood corpuscles into a suitable animal the haemolytic power of the serum may increase very rapidly and yet multiple doses of the immune-body may be deficient in the power of causing increased absorption of complement. On the other hand, in some animals both the haemolytic and the complement absorbing power rise rapidly.

Example. Date of experiment, 8. iii. '10.

Rabbit "FW," I.B. 1. iii. '10, 5 days after 1st injection (4 c.c. blood-sediment),

D. = 0.001 c.c.

" "S," I.B. 28. i. '10, 7 " " " " " D. = 0.0012 c.c.

Lysis of test corpuscles (1 c.c. + 5 D. of I.B.)

Amount of guinea-pig's complement in c.c. ....	1 D. of I.B.		4 D. of I.B.			
	0.015	0.02	0.01	0.02	0.035	0.055
"FW" just complete	complete	complete	0	very marked	complete	complete
"S" almost complete	just complete	complete	0	0	trace	almost complete

The immune-body in the serum of rabbit "S" at an earlier date after the first injection was deficient in complement-absorbing power, v. p. 214.

*The independence of the amount of complement necessary to cause lysis with an immune-body and the amount brought into combination through its agency.*

The independence of these two functions is well illustrated by the following experiment:—

Date of experiment, 19. v. '10.

Rabbit "B" 9. v. '10, 4 days after first injection (4 c.c. of blood-sediment),

D. = 0·035 c.c.

" 17. v. '10, 12 " " " " D. = 0·003 c.c.

Dose of mature I.B. = 0·0002 c.c.

Lysis of test corpuscles (1 c.c.+5 D. of I.B.)

Amounts of complement in c.c. ....	1 D. of I.B.			4 D. of I.B.			
	0·01	0·015	0·025	0·02	0·03	0·045	0·06
" B" 9. v.	almost complete	complete	complete	just complete	complete	complete	complete
" B" 17. v.	just complete	complete	complete	just complete	complete	complete	complete
Mature I.B.	trace	almost complete	complete	0	0	distinct	just complete

Initial lysis: most rapid with mature I.B., slowest with "B" 9. v.

" B" 9. v., 1 D. series + 0·025 c.c. complement = not complete.

" B" 17. v., 1 D. " + 0·01 c.c. " = just complete.

Mature I.B., 1 D. " + 0·01 c.c. " = complete.

Thus, while the complement is much more toxic with the specimen 17. v. than with 9. v. the amount of complement absorbed with both is practically equal and much less than what is absorbed by the mature immune-body.

*The combining power of immune-body with the red corpuscles.*

In several instances the combining power of immune-bodies at early stages of immunisation, for the red corpuscles was tested. We did not attempt to determine differences in the rate of combination, but allowed practically sufficient time for maximal combination.

The following is a representative experiment:—

Date of experiment, 22. ii. '11.

Rabbit "BH" I.B. 21. ii. '11, 5 days after 1st injection (2 c.c. of blood-sediment),

D. = 0·01 c.c.

Rabbit "GW" I.B. 21. ii. '11, 8 days after 3rd injection, D. = 0·0015 c.c.

Mature I.B. vii. '10, D. = 0·0007 c.c.

To a series of tubes each containing 0·5 c.c. blood suspension 3, 5, 7, 10 D. of I.B. were added, the tubes were then put at 37° C. for  $\frac{1}{4}$  hour and thereafter left for  $\frac{3}{4}$  hour at room temperature, being frequently shaken during this time. They were then centrifugalised and the fluid added to 0·5 c.c. blood suspension along with six doses of complement. The amount of lysis in the test corpuscles was read at the end of 1 $\frac{1}{4}$  hours at 37° C.

*Lysis of Test Corpuscles.*

Doses of LB.....	3	5	7	10
" BH "	very marked	almost complete	just complete	complete
" GW "	trace	very marked	almost complete	complete
Mature I.B.	very marked	complete	complete	complete

This result shows that provided at least sufficient time is given for combination, the rising immune-body shows no deficiency in its power of combining with the red corpuscles. The complement combining experiment carried out at the same time gave the usual result (Table IV).

TABLE IV.

Amount of complement in c.c. ....	Lysis of test corpuscles (0·5 c.c.+5 D. of I.B.)							
	1 D. of I.B.			5 D. of I.B.				
0 013	0 025	0 035	0 035	0 05	0 07	0 1	0 13	
" BH "	0·675	complete	complete	0·85	complete	complete	complete	complete
" GW "	0·5	0·9	complete	0·35	0·5	0·6	0·8	complete
Mature I.B.	0·5	0·95	complete	0·3	0·3	0·4	0·85	complete

This shows that the immune-body (" BH ") at the early stage has relatively weak powers of leading to absorption of complement when multiple haemolytic doses are used.

In the complement absorption experiments described above the immune-body was added to the corpuscles first and then the complement about 10 minutes later. Muir and Browning<sup>1</sup> had found in a certain case (ox corpuscles, immune-body from the rabbit and ox complement) that the resulting lysis was much more marked when the immune-body was added to the corpuscles a considerable time before the complement. Accordingly, we performed the following experiment to determine whether the length of time elapsing between the addition of the immune-body and the complement to the corpuscles affected the amount of complement absorbed in the case of a weak rising immune-body.

<sup>1</sup> *Vide Muir, Studies on Immunity.*

5 D. of I.B., Rabbit "BH" 21, n. '11, were added to series (*a*) and the mixture of immune-body and corpuscles was allowed to stand two hours at room temperature, then the same amount of I.B. was added to series (*b*) and the complement was added to both series immediately: lysis proceeded slightly more rapidly in series (*a*) than in series (*b*), but the end point was practically the same in both. After incubation for 1½ hours at 37° C. the uncombined complement was tested for in the usual way.

The result showed that the amount of lysis in corresponding tubes in both series was practically identical, that is to say, the amount of complement absorbed was not increased by allowing a long time for combination of the immune-body before adding the complement.

#### SUMMARY AND CONCLUSIONS.

(1) When rabbits are injected intraperitoneally with ox's red blood corpuscles the haemolytic immune-body which is developed shows qualitative differences at different stages of immunisation.

(2) The immune-body molecules which appear in the serum in the early stage of immunisation (*e.g.* four to eight days after a single injection of 2 to 4 c.c. of red blood corpuscles) are deficient in the power of causing absorption of complement when added to the corresponding blood corpuscles. This is most clearly brought out by the very slight increase in complement absorbed under the influence of multiple doses of immune-body as compared with the amount absorbed by one dose. The deficient complement absorption does not depend to any marked degree on deficient combination of immune-body with the receptors of the red corpuscles.

(3) When, after repeated injections of blood corpuscles, immunisation has been carried to such a stage that an immune-body is produced which is very active in causing absorption of complement, then it is found that on ceasing to give further injections the relative complement-combining power remains high after the immune-body content of the serum as measured by the haemolytic dose has fallen to a very considerable extent.

So far as we are aware the occurrence of such alterations in the properties of immune-body during the process of immunisation has not hitherto been noted.

## IMMUNITY OF GUINEA-PIGS TO DIPHTHERIA TOXIN AND ITS EFFECT UPON THE OFFSPRING.

### PART I.

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SINCE the time Ehrlich<sup>(1)</sup> established on a scientific basis many of the main facts regarding the transmission of immunity as exhibited in mice to the vegetable poisons, abrin, ricin and robin, many investigators have concerned themselves with the subject of transmission of immunity to various poisons and bacterial infections. Ehrlich first showed conclusively that the immunity transmitted from the mother was of a passive nature, due to the transference of the antibodies from the body fluids of the mother to the foetus through the placenta, and also in the case of the mouse by means of the milk during lactation. He found that the immunity thus transmitted disappeared some three months after birth; no trace of immunity was detected in the next generation; neither was an immunised father capable of transmitting immunity to his offspring. Ehrlich concluded from these results that there exists no true hereditary transmission of immunity in mice, a conclusion further supported in both mice and guinea-pigs immunised against tetanus<sup>(2)</sup>.

These results have in the main been confirmed by all later observers. The fact that the young of mice become immunised through the milk is exceptional and not generally shown by other animals experimented upon. Carrière<sup>(3)</sup> obtained results in guinea-pigs inoculated with products of the tubercle bacillus which show that not only is immunity in the strict sense of the term not transmitted to the offspring but on the contrary a condition of increased susceptibility and a general lowered condition of vitality of the offspring resulted.

Lustig<sup>(4)</sup> similarly demonstrated that fowls inoculated with abrin produce offspring of less than normal resistance to this poison.

Anderson<sup>(5)</sup> appears to be the first to have shown that the young of guinea-pigs treated with a mixture of diphtheria toxin and antitoxin exhibit immunity to diphtheria toxin, no such immunity being shown in the young of those treated with toxin only. The immunity was not transmitted by all mothers treated with the toxin-antitoxin mixture, and this he compared with the fact that horses respond very differently to diphtheria toxin in their capacity to produce antitoxin. Grandchildren of immune mothers were not more resistant than normal. Theobald Smith<sup>(6)</sup> arrived independently at results confirming those of Anderson.

In a second paper Theobald Smith<sup>(7)</sup> arrived at the following conclusions:

Active immunity to a relatively high degree can be produced in guinea-pigs by the injection of diphtheria toxin-antitoxin.

Neutral mixtures are less effective in producing immunity than mixtures which contain excess of toxin capable of giving rise to local lesions.

We have been engaged on observations on somewhat similar lines for a number of years. We have worked on a larger aggregate of material, and by methods differing in some important particulars from those adopted by the above observers. Although, therefore, our results to a large extent correspond with those which have been published meanwhile by others independently engaged with the question, we think it desirable to place them on record. We have, however, delayed publication until various other questions which had arisen in connection with certain phenomena occurring in the second and third generation could be followed out.

Our method of determining the degree of immunity conferred on the offspring differed from that of Theobald Smith who ascertained the L<sub>+</sub> dose on animals of a definite age, while our results were based upon the effect of the injection of toxin only as soon as the young had reached the weight of 250 grams. The degree of immunity depending presumably upon the quantity of circulating antitoxin is more accurately expressed by the neutralising value in terms of the volume of toxin (indicating the number of binding units) rather than the number of fatal doses tolerated. In all cases tabulated the same toxin (98 A) was used throughout.

The seasonal variation<sup>(8)</sup> of susceptibility of guinea-pigs which affects so markedly the apparent minimal fatal dose of a toxin is barely notice-

able in testing neutralising values. In the same way the volume of toxin tolerated by immune young (*i.e.* neutralised by their antitoxin) shows no great variation at different times of the year except in those cases where the volume of toxin tested was very close to the normal fatal dose. In testing the immunity of a pig only one test is permissible, so that it is seldom possible to determine accurately the immunity value, beyond saying that it is above or below a certain value. When there are three or four pigs in one litter, the first pigs reaching the standard weight can be used as an index for the value to test for in the other pigs of the same litter, and so a more accurate immunity value can be obtained.

In tabulating the results of immunity conferred upon their young by immunised does the degree of immunity is measured by the largest dose of toxin tolerated by any of the offspring, or if the doses given were in all cases too high and none of the young survived, the immunity is taken to be less than the lowest dose that killed. In practice it was found generally unworkable to investigate a particular point by performing definite experiments avoiding all other variables. The possibilities of the injected pigs dying before any young were born, or of the young dying before they reached standard weight, or of there being an insufficient number of young to fix the degree of immunity with any accuracy, were so great that it was found best to obtain our main results by keeping isolated and breeding from a large number of guinea-pigs that survived injections of toxin or toxin and antitoxin mixture given in the course of the regular laboratory routine rather than by specially injecting a limited number of pigs and endeavouring to breed from them. The results obtained in testing the immunity of the offspring from these pigs could then be tabulated. In each set of tables where the results are divided according to a particular variable the other variables are ignored. When it would appear that the results of one variable would overwhelm those of another, individual cases are dealt with.

The first part of our results deals only with the first generation of the young from injected does and normal bucks.

A. *Immunity transmitted to young by mother injected with various mixtures of toxin and antitoxin.*

The variables upon which the immunity of the young may depend are:

1. Nature of the injection received by the mother.
2. Individual differences of different mothers.

3. Time elapsing between injection of mother and birth of young.
4. Age of young when tested for immunity.
5. Time of year and climatic conditions when test was made.
6. Individual differences in young.

These variables are dealt with individually below :

*1. Nature of injection received by mother.*

*1 a.* The constitution of the mixture and the degree to which the mother was affected may be indicated by the local reaction produced by the injection. In Table I the results are divided according to whether the local reaction produced was nil, small or large. The results of immunity conferred by the mother are indicated by the largest dose of toxin tolerated by any of their young, or if none of the young survived the dose given, then the lowest dose that killed is recorded, provided that death occurred within 10 days of the test and that the test was made upon young born within 12 months of the injection of the mother. It should be remembered that in these tables each mother is represented only once although tests may have been made upon many of its offspring.

TABLE I.

*Results of immunity of young divided according to the local reaction produced by the immunising mixture when injected into the mother.*

			Size of local reaction in mother		
			Nil	Small	Large
Lowest dose killing young	Under 0·008 c.c.	...	4	2	0
	0·008 and under 0·010 c.c.		9	2	0
	0·010 ,,, 0·020 c.c.		8	1	1
	0·020 ,,, 0·030 c.c.		2	0	0
Highest dose tolerated by young	0·008 and under 0·010 c.c.		3	0	0
	0·010 ,,, 0·020 c.c.		6	3	6
	0·020 ,,, 0·030 c.c.		4	1	3
	Over 0·030 c.c.	...	3	2	2

To summarise these results it is necessary to consider the proportion of does whose young tolerated a particular dose or more of toxin to those whose young were killed by that or a lower dose of toxin. Thus in Table I of the mothers showing nil swelling, eight had young killed by a dose of toxin of 0·010 to 0·020, nine had young killed by a dose

between 0·008 and 0·010 and four below 0·008, therefore the young of these 21 would all have died if injected with 0·020 and in the same way seven had young capable of surviving an injection of 0·020 c.c.

TABLE II.

Dose at or above which young survived and at or under which young died	Size of local reaction in mother					
	Nil		Small		Large	
	Survivals	Deaths	Survivals	Deaths	Survivals	Deaths
0·008 c.c.	16	4	6	2	11	0
0·010 c.c.	13	13	6	4	11	0
0·020 c.c.	7	21	3	5	5	1
0·030 c.c.	3	23	2	5	2	1

TABLE III.

*Showing the percentage numbers of mothers whose young survived any particular doses.*

Dose	Size of local reaction in mother		
	Nil	Small	Large
0·008 c.c.	80	75	100
0·010 c.c.	50	60	100
0·020 c.c.	25	37	83
0·030 c.c.	11	28	66

From Table III it is seen that mixtures nearer L+ than L0 confer higher immunity—the young of over 80% of the does injected with toxin-antitoxin mixtures producing large local reactions tolerated 0·020 c.c. of toxin, while the young of only 25% of the does injected with mixtures producing no local reaction tolerated the same dose. It should be pointed out that these tables represent the results from 62 mothers but at least 400 young from these mothers were tested. A somewhat noticeable exception to the figures occurs in the case of two mothers with small local reactions whose young died with doses less than 0·008 c.c. In both cases the pigs were used for testing very weak antitoxic serum and were injected with a test dose of toxin together with  $\frac{1}{2}$  c.c. of serum. This quantity of serum in itself is enough to account for a small local reaction on the pig. In both cases the rapid growth of the pigs after the injection indicates that so far as the toxin reaction is concerned the swelling should be considered as nil. Table IV gives the results as they would read if this alteration were made.

1 b. Probably the most essential feature of the toxin-antitoxin mixture injected into the mother is the amount of toxin (or toxoid)

dissociated, and this is best indicated by the excess of antitoxin present in the mixture above that necessary to protect life until the 5th day when injected with one test dose of toxin. In many cases the mothers had been used for giving only a rough approximation of the antitoxic

TABLE IV.

Dose	Size of local reaction in mother		
	Nil	Small	Large
0·008 c.c.	72	100	100
0·010 c.c.	46	75	100
0·020 c.c.	23	50	83
0·030 c.c.	10	40	60

value of a serum and so the exact value of the serum used was not known in all cases. The figures in Tables V, VI and VII give the results for all mothers where it was known that the excess of antitoxin in the mixture was less than 20%, less than 50%, or over 50%.

TABLE V.

*Results of immunity of young divided according to the excess of antitoxin present in the toxin-antitoxin mixture injected into the mother.*

		Excess of antitoxin		
		Over 50%	Under 50%	Under 20%
Lowest dose killing young	Under 0·008 c.c.	...	2	1*
	0·008 and under 0·010 c.c.		3	1
	0·010	0·020 c.c.	3	2
	0·020	0·030 c.c.	1	0
Highest dose tolerated by young	0·008 and under 0·010 c.c.		0	0
	0·010	0·020 c.c.	2†	7
	0·020	0·030 c.c.	0	2
	Over 0·030 c.c.		0	3

\* This pig was paralysed and consequently its responsive power was impaired.

† One of these figures is the result of only a single test upon one young and may be a case of accidental survival through leakage of injection. The other refers to a pig that gave a most unusual result of no local reaction but a marked drop in weight, indicating that the test was "out" in some way.

TABLE VI.

Dose at or above which young survived and at or under which young died	Excess of antitoxin					
	Over 50%		Under 50%		Under 20%	
	Survivals	Deaths	Survivals	Deaths	Survivals	Deaths
0·008 c.c.	2	2	12	1	5	0
0·010 c.c.	2	5	12	2	5	0
0·020 c.c.	0	8	5	4	2	0
0·030 c.c.	0	9	3	4	0	0

TABLE VII.

*Showing the percentage numbers of mothers whose young survived any particular dose.*

Dose	Excess of antitoxin		
	Over 50 %	Under 50 %	Under 20 %
0·008 c.c.	50	92	100
0·010 c.c.	28	85	100
0·020 c.c.	0	55	100
0·030 c.c.	0	42	—

Table VII shows very definitely that the immunity conferred by a doe upon the young depends upon the amount in the toxin-antitoxin mixture of toxin (or toxoid) dissociated and so available for immunisation. In the case of eight does where the excess of antitoxin was greater than 50 % none of their young could tolerate 0·020 c.c. of toxin, while the young from five out of nine mothers receiving under 50 % excess of antitoxin tolerated this dose.

1c. Another variable in the constitution of the mixture injected into the mother is the constitution of the toxin used. The majority of the results recorded were from mothers injected with the same test toxin but in the few cases where other toxins were used there was a strong indication that a fresh toxin does not give such high immunity as an older toxin where there is more toxoid dissociated in a neutral mixture. The results obtained are too few to form any definite conclusion unless all other variables can be eliminated.

1d. Another variable in the constitution of the mixture is the amount of toxin injected. The results recorded so far are from cases where the mother received one test dose of toxin together with antitoxin. In testing the antitoxic value of the serum obtained from apparently normal horses it is often necessary to supply 1/5 or 1/10 of the test dose of toxin. The young bred from mothers used for these tests did not as a general rule give very high immunity. In one case a moderately high degree of immunity was shown, this however was not to be compared with the highest degrees of immunity reached by the young of mothers treated with a full test dose of toxin.

## 2. Individual differences in parents.

In most animals individuals of the same species appear to vary in their responsiveness to immunisation. Various slight irregularities in the foregoing tables are sufficient to show that such variability does exist in guinea-pigs. A definite example is given below.

Two pigs 100 Z and 105 AA were injected with a test dose of the same toxin and 1·3 unit antitoxin. Nine months after the injection the young were tested with 0·015 c.c. toxin, that of 100 Z gave no local reaction, while that of 105 AA gave a very large local reaction and a drop of 35 grams in weight on the fifth day after injection. Subsequent tests of later litters showed that the young of 100 Z would tolerate 0·050 c.c. of toxin better than those of 105 AA would tolerate 0·015 c.c.

### 3. Time elapsing between injection of mother and birth of young.

In the majority of cases the young of the first and possibly the second litter were tested with doses well above or well below the dose they would just tolerate, consequently very few examples illustrate the degree of immunity at all stages in the life of the mother. That a gradual diminution in immunity or at least in the power of transmitting immunity occurs in the mother is shown in the individual cases given in Table VIII.

TABLE VIII.

Cage	Litter	Time elapsing between injection of mother and birth of young	Age of young	Dose injected	Date of death	Local reaction	Change in weight
47 A	1st	13 months	32 days	0·016 c.c.	...	Very large	- 35 grm.
			37 "	0·018 "	...	"	- 50 "
	2nd	20 "	35 "	0·006 "	...	"	- 15 "
			35 "	0·010 "	6th day	...	...
105 B	1st	5 "	40 "	0·022 "	...	Very large	- 30 grm.
			44 "	0·024 "	...	"	- 55 "
			44 "	0·026 "	...	"	- 45 "
	2nd & 3rd	...	Still borns	...	...	...	...
	4th	16 months	56 days	0·018 c.e.	3rd day	...	...
			56 "	0·015 "	4th "	...	...
153 F	1st	6 "	42 "	0·012 "	...	Very large	+ 10 grm.
			42 "	0·021 "	...	"	- 10 "
	2nd	...	Still borns	...	...	...	...
	3rd	14 months	35 days	0·028 c.e.	4th day	...	...
			35 "	0·025 "	5th "	...	...
153 AA	1st	9 "	18 "	0·015 "	...	Very large	- 55 grm.
	2nd	12 "	32 "	0·008 "	6th day	...	...
94 B	1st	11 "	38 "	0·020 "	...	Very large	- 5 grm.
	2nd	14 "	35 "	0·016 "	...	"	- 15 "
			42 "	0·018 "	6th day	...	...
	3rd	20 "	50 "	0·015 "	4th "	...	...
			61 "	0·012 "	5th "	...	...
4th	25 "	21 "	0·008 "	...	Very large	- 20 grm.	
		28 "	0·008 "	...	"	- 10 "	

The cases recorded in Table VIII show more or less rapid loss of immunity in the mother, but they cannot be closely analysed in consequence of the variation of immunity according to the age of the young which is to be dealt with next.

We have demonstrated immunity to the extent of tolerating 10 fatal doses of toxin in young born 25 months after the injection of the mother.

#### 4. *The age of the young when tested for immunity.*

In all cases the young are tested when they have reached the weight of 250 grams, the time taken for pigs to reach this weight varies from 10 days to 120 days. Tables IX, X, XI, give the results of over 300 young whose exact ages were known. It should be noted that these tables differ from I, II, III and V, VI, VII as all the young are given irrespective of the treatment of the parents while the earlier tables recorded the mothers and not the young.

TABLE IX.

		Age of young in days						
Dose tested		20 or less	21-30	31-40	41-50	51-60	Over 60	
Deaths	Under 0·008 c.c.	...	2	4	2	1	7	14
	0·008 and under 0·010 c.c.	4	21	21	12	4	1	
	0·010	,,	0·020 c.c.	4	12	12	13	6
Survivals	0·020	,,	0·030 c.c.	5	7	2	9	5
	0·008 and under 0·010 c.c.	9	10	4	1	1	0	
	0·010	,,	0·020 c.c.	8	17	18	9	3
	0·020	,,	0·030 c.c.	1	7	9	4	5
Over 0·030 c.c.		...	1	3	4	3	0	0

TABLE X.

Doses at or above which young survived and at or under which young died	Age of young in days											
	20 or less		21-30		31-40		41-50		51-60		Over 60	
Sur- vivals	Deaths	Sur- vivals	Deaths	Sur- vivals	Deaths	Sur- vivals	Deaths	Sur- vivals	Deaths	Sur- vivals	Deaths	
0·008 c.c.	19	2	37	4	35	2	17	1	9	7	1	14
0·010 c.c.	10	6	27	25	31	23	16	13	8	11	1	15
0·020 c.c.	2	10	10	37	13	37	7	25	5	24	0	21
0·030 c.c.	1	15	3	44	4	41	3	27	0	33	0	26

Table XI shows that immunity has almost disappeared at the end of two months. But that it may occasionally be present, however, to a considerable extent after this period will be shown later when we record cases in connection with another section of the subject.

TABLE XI.

*Showing the percentage number of young of different ages that survived any particular doses.*

Dose	Age of young in days					
	20 or less	21-30	31-40	41-50	51-60	Over 60
0·008 c.c.	90	90	94	94	56	6
0·010 c.c.	62	51	57	55	47	6
0·020 c.c.	16	21	26	21	17	0
0·030 c.c.	6	6	8	10	0	0

Table XII gives individual cases of the effect of the age of the young.

TABLE XII.

Cage	Time elapsing between injection of mother and birth of young	Age of young	Dose injected	Date of death	Local reaction	Change in weight
47 B	14 months	51 days	0·009 c.c.	3rd day	...	...
		58 "	0·007 "	5th "	...	...
	17 "	52 "	0·006 "	3rd "	...	...
		73 "	0·0055 "	...	Very large	-45 grm.
		28 "	0·008 "	...	"	-45 "
100 N	9 "	50 "	0·015 "	15th "	...	...
		31 "	0·020 "	...	Very large	-15 grm.
	14 "	56 "	0·030 "	7th day	...	...
105	6 "	56 "	0·030 "	7th day	...	...
		70 "	0·030 "	3rd "	...	...
	6 "	29 "	0·026 "	...	Large	+25 grm.
106 D	9 "	60 "	0·020 "	...	Very large	-25 "
		36 "	0·020 "	...	"	+25 "
	14 "	29 "	0·025 "	...	Very small	No change
106 L	6 "	60 "	0·025 "	...	Large	-10 "
	11 "	29 "	0·025 "	...	Very small	No change
153 A	5 "	50 "	0·018 "	...	Very large	-10 grm.
		87 "	0·018 "	3rd day	...	...

It will be seen from Table XII that in some cases, notably in cage 100 N, young pigs of subsequent litters will tolerate a larger dose than older pigs of earlier litters owing to the more rapid loss of immunity in the young compared to that in the mother. This result is what would have been expected from the fact that passive immunity is more transient than active. The smaller dose tolerated by older pigs is due to loss of antitoxin and not to any weakness of the pig indicated by the longer

time taken to reach the standard weight, because we have shown(s) that for normal guinea-pigs of the same weight the minimal fatal dose increases with age. This is also seen in Table XIII giving the time taken by 0·008 c.c. toxin to kill normal guinea-pigs of different ages during the months of May, June and July, 1909.

TABLE XIII.

	Age of guinea-pigs		
	20-30 days	31-40 days	41-50 days
Time taken to kill	3 days	3 days	4 days
	3 "	3 "	7 "
	3 "	4 "	11 "
	4 "	6 "	
	4 "		

#### 5. Time of year and climatic conditions.

This has been shown to have a marked effect on normal guinea-pigs, and a similar effect was noticeable on young showing little or no immunity. The effect has not been demonstrated clearly in the case of young showing considerable immunity. The effect would be quite masked by other variables in any attempt to show the effect by statistical methods.

#### 6. Individual differences in young.

Throughout our work there has been evidence that slight individual differences do occur, but great variations in immunity have not been observed. The differences noted were small and mostly to be accounted for by the age of the pigs. It seldom happened that a direct comparison could be made on different individuals of the same litter reaching the standard weight on the same day. In one case however (eage 105 TT) three pigs of the same litter were tested when 35 days old and 250 grams in weight. The dose used was 0·015 c.c. toxin and the results were

Doe ... ... died in three days.

Buck ... ... " " seven "

Buck ... ... Survived with very large local reaction and a loss of 50 grams in weight on the fifth day.

*General notes on results obtained with young from mothers injected with toxin-antitoxin mixtures.*

Results obtained with cage 19 afford a very good series showing the combined influence of the age of young when tested and the time elapsing between injection of mother and birth of young. The figures are given in Table XIV.

TABLE XIV.

Litter	Time elapsing between injection of mother and birth of young	Age of young	Dose injected	Date of death	Local reaction	Change in weight
1 }	All still born	...	...	...	...	...
		...	...	...	...	...
2 }	11 months	49 days	0·018 c.c.	...	Large	- 5 grm.
		56 "	0·020 "	4th day	...	...
3	14 "	57 "	0·014 "	...	Very large	- 25 grm.
		62 "	0·016 "	4th day	...	...
4	19 "	70 "	0·010 "	...	Very large	- 10 grm.
		84 "	0·012 "	5th day	...	...
5	22 "	34 "	0·012 "	...	Large	+ 15 grm.
		45 "	0·015 "	...	,,	- 15 ,,

In each litter the results are sufficiently clear to enable us to fix a fatal dose as an index of the immunity possessed by the young of that litter.

TABLE XV.

Litter	Time elapsing between injection of mother and birth of young	Average age of young	Minimum fatal dose for litter
3	11	52½ days	0·019 c.c.
4	14	59½ "	0·015 ", -
5	19	77 "	0·011 ", +
6	22	39½ "	0·016 ",

From Table XV it will be seen that the degree of immunity of each litter is in order of the average age of the young of that litter if allowance is made for a slight decrease owing to loss of immunity in mother.

As would be expected the amount of different toxins tolerated by immune young depends not upon the number of fatal doses but upon the binding units. Immune young will tolerate many more fatal doses of a fresh toxin containing little toxoid than of an old toxin rich in toxoids.

The results recorded so far were made with toxin (98 A) of which the average fatal dose for the year could be taken as 0·008 c.c. Another bottle (98 B) of the same toxin was found to have diminished more rapidly in toxicity so that at the time of testing (eight years after the preparation of the toxin) the average fatal dose was 0·014 c.c. It was found that the volumes of 98 A and 98 B tolerated by highly immune young were almost identical. The highest number of fatal doses that immune young survived when tested with 98 A was 7, with another toxin (967 A) the highest number was 14. In volume the highest amounts tolerated were similar and the binding unit content of the two toxins were similar although the fatal dose of 98 A (eight years old) was 0·008 c.c. and that of 967 A (two years old) was 0·004 c.c.

B. *Immunity transmitted to young by mother injected with toxin only.*

It has already been demonstrated clearly by Anderson and Theobald Smith that single injections of sub-lethal doses of toxin in does will not produce immunity in the young. A set of six does was put aside for breeding to confirm this and in no case could we demonstrate immunity in the young. A further set of three does was treated with a series of gradually increasing doses of toxin and again no immunity could be detected in the young. Two of the does treated in this way received similar injections (1/5, 2/5, 2/3 and 1 M.F.D. at weekly intervals). At the end of the treatment one (100 A) had decidedly decreased in weight and the other (100 B) increased. In the case of 100 A only one out of seven young from the first two litters survived while in 100 B four out of six lived. The young of 100 B all showed normal susceptibility to toxin but those of 100 A showed a lowering of resistance as seen in Table XVI. The comparison between the two pigs is shown in Table XVII.

Lustig has shown that immunity to abrin is not transmitted by fowls to their offspring. We shall similarly show in a subsequent section of this investigation that there is evidence against transmission of immunity to diphtheria toxin by the effect on the germ cell before fecundation. Immunity in the young must depend therefore upon the transference of passive immunity from the mother either during pregnancy or by means of the milk. Several experiments were made but only one case was recorded in which a normal pig suckled by a highly immune doe survived the normal lethal dose. In two cases out of three there

appeared to be very slight lowering of the immunity when immune young were suckled by normal does. Thus it appears that immunity is mainly transferred through the placenta during pregnancy. A few experiments were performed to see whether immunity passively acquired by the mother could be so transferred. In eight cases where does were injected at different times during the course of pregnancy with 2000 units of diphtheria antitoxin: no immunity could be detected in the young. This failure may be due to the more rapid loss of passive immunity conferred by the inoculation with antitoxin obtained from another species of animal.

TABLE XVI.

Young from 100 A		Normal young	
Dose	Date of death	Dose	Date of death
0.008 c.c.	5th day	0.009 c.c.	4th day
0.009 ..	3rd ..	0.009 ..	5th ..
0.009 ..	3rd ..	0.009 ..	5th ..
		0.009 ..	6th ..

TABLE XVII.

Pig	Change in weight during treatment	Resistance of young to Diphtheria toxin	Percentage sur- vivals in first two litters	Percentage survival during total time of breeding
100 A	- 20 grm.	Lowered	14	46
100 B	+ 40 ..	Normal	66	75

## SUMMARY.

1. Diphtheria toxin-antitoxin mixtures induce a higher immunity in guinea-pigs than sub-lethal doses of toxin; one injection of the mixture being sufficient to produce an immunity lasting in some cases for a period of over two years, as shown by the passive immunity conferred on the offspring.
2. The highest immunity is produced by toxin-antitoxin mixtures containing the most uncombined toxoid.
3. The active immunity of the mother is transferred passively to the offspring.
4. The passive immunity thus transferred usually disappears at the end of two months after birth, and only in rare instances has been recognised after three months.

5. Immunity is mainly transmitted in utero, and only to a slight extent during lactation.

6. Young bred from does that have been used for a single routine antitoxin test may be able to tolerate 14 times the dose of diphtheria toxin fatal for a normal guinea-pig.

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## THE SAND FILTRATION AND PURIFICATION OF CHALK WATERS.

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(1 Chart.)

SOME deep wells in the chalk are liable to contamination. The pollution may gain access to the wells by means of fissures or "swallow-holes" in the water-bearing stratum, and, through these defects, micro-organisms and organic matter may find their way into the water supply of a town from points many miles distant. The risk of chalk waters becoming contaminated is daily growing more probable: quick and cheap transit is responsible for an exodus from the town to the country which serves as a gathering ground for water supplies; and the chalk uplands and countryside, which formerly were sparsely populated, are in many places becoming covered with collections of houses and small villages, whose only method of sewage disposal is by means of cesspools into the chalk on which the houses are built. If the chalk is sound and unfissured, this method of sewage disposal is not harmful to distant water supplies; but, given a fissure or swallow-hole, the intestinal micro-organisms and organic matter from some outlying village, may make the water from a deep well some miles away unfit to drink. I know at present of five important wells in the chalk in different parts of England, which are, in this manner, liable to intermittent and undoubted faecal contamination; but, in only two of these places are means taken to purify the water before it is distributed to the consumers. A typhoid carrier or a case of enteric fever might distribute bacilli to a distant town through some imperfection in the stratum, and the consequences, without doubt, would be very disastrous; yet probably there

are many towns in the country which take the risk of such an epidemic.

One of the objects of this paper is to insist on the necessity of frequent bacteriological examinations of chalk water supplies; so that, when intermittent contamination is known to occur, some method may be adopted of purifying the water.

*Scope of the present enquiry.*

During the first four months of this year I conducted bacteriological researches on a deep well in the chalk, and on three filter beds and two Porter Clark softening plants, by which this well water is treated before distribution.

My object was to gain information into the bacteriology of the well water at different times, to determine whether or not the sand filtration was effective, and to see whether or not the softening of a chalk water by the rapid Porter Clark process caused any purification from micro-organisms. I shall now consider these three main divisions of my work.

*The well and the well water.*

The well is 200 feet deep, and has a delivery of about 700,000 gallons per day of 10 hours. The water is pumped directly from the well to the filter beds, and thence to a reservoir of five million gallons capacity.

The well is subject to intermittent contamination from swallow-holes. Some years ago an organism, foreign to the well water, was recovered from the well, after having been put down in large numbers and washed into the soil in the neighbourhood of one of these swallow-holes. Sodium chloride and fluorescein have, in a similar way, been used in the past to demonstrate the connection between these distant swallow-holes and the well. There is no doubt that the well sometimes becomes foul. This, however, rarely happens more than five times a year. Half an inch to an inch of rain in 24 hours causes the well water to become muddy, and on such occasions the bacterial count is very largely increased.

Normally the well water is very pure. *Bacillus coli* does not occur in 100 c.c. *Bacillus enteritidis sporogenes* is not found. Only a few colonies develop on agar in 24 hours, and about a couple of dozen per c.c. on gelatin in 72 hours. Gelatin liquefiers are few in number. The

average count of the well water under normal conditions is seen in the following table:

TABLE A.

*Average number of Bacteria in well water—excluding days on which the well was contaminated.*

<i>B. coli</i> per 100 c.c.	...	...	...	= 0·8
Colonies developing on agar at 37° C. in 24 hours	...			= 4·12 per c.c.
Colonies on gelatin 20—22° C. in 72 hours	...		= 24·1	„
Gelatin liquefiers in 72 hours	...	...	= 0·7	„

On two occasions during my four months' work I have found the well to be contaminated. The number of organisms increased very greatly. Tables B and C show the bacterial count at these times.

TABLE B.

*Average number of bacteria in well water, during contamination in January 1911.*

<i>B. coli</i> per 100 c.c.	...	...	...	= 10·5
Colonies developing on agar at 37° C. in 24 hours	...			= 97·1 per c.c.
Colonies developing on gelatin at 22° C. in 72 hours	...		= 125·7	„
Gelatin liquefiers in 72 hours	...	...	= 14·9	„

TABLE C.

*Average number of bacteria in well water, during contamination in April 1911.*

<i>B. coli</i> per 100 c.c.	...	...	...	...	= 25
Colonies developing on agar at 37° C. in 24 hours	...				= 383 per c.c.
Colonies developing on gelatin at 20—22° C. in 72 hours	...				= 1213 „
Gelatin liquefiers in 72 hours	...	...	...		= 206 „

*Note:—*In Tables B and C the figures are averaged from several days observations.

#### *The filter beds.*

There are three filter beds in connection with this well. Each has an area of one-third of an acre. They are immersed and uncovered beds of the ordinary type. The top sand is two feet in thickness and is fine—50 per cent. will pass a “30 × 30 Sieve.” The head of water required to work the beds is only about  $1\frac{1}{2}$  inches. The water flows through the beds at the rate of nearly four inches an hour.

*The growth of Algae.*

Many difficulties have arisen in connection with the working of these beds—the chief and most important, from the point of view of the owners of this water supply, being the fact that the beds become overgrown with green algae, which decompose rapidly and make the filtered water offensive both to taste and smell. In consequence there have been many complaints from the consumers of the water. When the beds are emptied to be cleaned the remains of the decaying weed are found on the sand. This dead organic matter looks like freshly-passed cow-dung, and smells very foul: so foul is it indeed, that the men who clean the beds say openly that they would rather be working on the sewage farm and cleaning out the septic tanks. These dung-like deposits and the upper inch of the sand are full of the larvae of *Chironomus*. Such is the material through which the water is filtered before it passes to the consumers.

For a week after cleaning one of these filter beds, no green weed is visible. Then it begins to grow vigorously; and, in a week, the bed is full of it. The more the sunlight, the more the weed. Three weeks from the time of cleaning, the water may begin to smell. In a month it may be intolerable, and the bed will have to be cleaned again. In sunless weather the bed may run as long as three months without cleaning.

*The "vital layer."*

No "vital layer" forms on the filter beds. It seems that a chalk water is too pure to provide the organic matter necessary for the formation of the layer. The sand, except where it is covered with masses of decomposing algae, remains as free from a vital layer as it was on the day the filter was first used. This seems to be one of the main difficulties in the sand filtration of chalk waters—a vital layer will not form on the filter beds.

*The efficiency of sand filtration of chalk waters.*

As I have said, the well water is very pure—except on a few days in the year. It can therefore hardly be thought surprising that the water should come from the filter beds more impure bacterially than it enters them. And such is the case. The filter beds certainly abstract some

organisms from the entering water; but at the same time they add other organisms.

The following table shows the bacterial content of the water before and after filtration. The figures are averaged from many observations extending over four months' work, and are exclusive of the high counts observed during the two periods when the well was contaminated.

TABLE D.

	<i>B. coli</i> per 100 c.c.	Colonies develop- ing on agar at 37° C. in 24 hrs.	Colonies on gelatin at 22° C. in 72 hrs.	Gelatin liquefiers
Unfiltered water	0·8	4·12 per c.c.	24·1 per c.c.	0·7 per c.c.
Filtered water	0·27	13·6 , ,	150·0 , ,	37·0 , ,

Now, the addition of some organisms to a water does not necessarily make this water less potable; and, in my opinion, the organisms added by these sand filter-beds do not make the water harmful to the consumers. The point to be settled was, did the beds effect a large reduction of the organisms present in the well water: or, to put the matter in other words, would the filter beds purify the well water, if the latter was heavily infected with—say—the *Bacillus typhosus*?

#### *The sand filtration of an artificially infected chalk water.*

To settle this question I determined to infect the water before filtration with some non-pathogenic organism that could be identified easily; and I came to the conclusion that a non-pathogenic variety of *Bacillus coli* would be the most convenient. In its shape and mobility it approaches *B. typhosus*, the organism against which we attempt to secure our water supplies. I had obtained a sucrose-fermenting *B. coli* from the well on a former occasion, and prepared a gallon of sterile peptone water into which I inoculated a pure culture of the Bacillus. Before adding any of the peptone water culture to the filter beds, I drank a pint of water heavily infected with this variety of *B. coli* with no evil results. *B. coli* is most useful as a Test Organism, not only from the fact that it is something like *B. typhosus*, but also because its presence is detected so easily and certainly. In passing I may say, that in isolating the organism subsequently I used neutral-red-bile-salts-peptone-lactose-agar,—the Rebipelagar of Houston,—and lactose-bile-salts-neutral-red-peptone water. Many thousand colonies were counted in all, and several hundred sub-cultured. The peptone water culture for infecting the

filter beds was put through the various sugars and plated on Rebipelagar and Conradi-Drigalski media: it was always determined that this culture was pure before it was used.

The culture of *B. coli* in peptone water was introduced into the filter beds by a long siphon-tube discharging the culture at the mouth of the water inlet pipe. The culture was thus delivered fairly uniformly, and well mixed with the inflowing water. Half-hourly samples were taken from the water at the far side of the bed; and half-hourly samples were taken of the filtered water. *B. coli* was not present in the filtered water before the experiment: it was generally present in the filtrate an hour from the time the culture was first added.

The following table shows the numbers of *B. coli* present in the water before and after filtration. The figures are averages. For further details the reader is referred to Appendix I, at the end of this paper.

TABLE E. *Filter A.*

Average number of <i>B. coli</i> in water before sand filtration	33.5 per c.c.
" " " after "	3.9 "
Percentage reduction brought about by sand filtration ...	88.4 %

The above figures refer to Filter A which had been cleaned out a fortnight previously.

A week later I performed a similar experiment on Filter C which had not been cleaned for nearly three months. The following results were obtained; details of which are given in Appendix II.

TABLE F. *Filter C.*

Average number of <i>B. coli</i> in water before sand filtration	40.1 per c.c.
" " " after "	1.28 "
Percentage reduction brought about by sand filtration ...	96.9 %

The conditions of these two beds were similar, except in the matter of cleaning. It would seem therefore that the dead organic matter and débris on the second bed acted in some way in place of the vital layer, and caused this bed to be a more effective filtering agent than its more recently cleaned fellow.

I am of opinion that the amount of purification these beds give is not sufficient for safety. That many of the added *B. coli* passed into the town supply is unquestionable, and I do not doubt that, if the organism had been *B. typhosus*, the results would have been deplorable.

*The formation of an artificial "vital-layer."*

Seeing that the mere sand filtration of a chalk water gave unsatisfactory results, probably on account of the lack of a vital layer on the filter beds, I decided to try the effect of a coating of aluminium hydrate on the surface of the sand, so as to simulate as closely as possible the absent vital layer. The aluminium hydrate was obtained by adding aluminium sulphate to the water as it entered the filter bed. The requisite quantity of the sulphate was suspended in a sack at the mouth of the inlet pipe; and, as the bed was filled, a gelatinous precipitate of the hydrate was deposited over the sand. The chemical reaction that takes place depends on the temporary hardness of the water, and can be expressed as follows:



The amount of aluminium sulphate that can be precipitated by a chalk water depends therefore, according to the equation, on the amount of temporary hardness present in the water. If excess of aluminium sulphate is added, it will pass into solution and be detected by the consumers. Practically, for each degree of temporary hardness, two-thirds of a grain of aluminium sulphate can be added to each gallon. The water on which I have been working has a temporary hardness of about 12° Clark's scale. I could add therefore with safety eight grains of aluminium sulphate to the gallon of water, knowing that all would be precipitated on the surface of the filter bed. I added however only one grain to the gallon while the bed was filled after cleaning; but continued the addition daily of a like amount during five days. At the end of this time there was a visible precipitate over the surface of the sand.

I then proceeded to infect the bed with *B. coli* in the same way as beds A and C. *B. coli* was added to the incoming water, and samples were taken of this water before and after filtration. The following table gives the results obtained. For further details the reader is referred to Appendix III.

TABLE G.

Average <i>B. coli</i> before filtration through aluminium hydrate and sand	73.9 per c.c.
Average <i>B. coli</i> after	0.4 "
Percentage purification brought about by filtration through precipitated aluminium hydrate and sand .. . . . .	99.46 %

It will be seen therefore that the purification obtained by filtering through precipitated aluminium hydrate and sand is considerably better than is given by filtration through sand alone.

The growth of algae, however, was not prevented by the precipitated aluminium hydrate. There was growth at the end of a week; and, at the end of a fortnight, the weed was rising to the surface and, doubtless, disturbing the layer of aluminium hydrate on the top of the sand. Three weeks after the first infection of the bed, I infected it again to see whether or not its powers of purifying the water from *B. coli* had been impaired by the growth and movement of the weed. The bed gave a purification of 99·21 %—showing only a slight diminution of its efficiency. I repeated the experiment again at the end of another three weeks, during which interval there had been increased growth and movement of algae. The percentage purification given by the bed was diminished, only 97·64 % of the added *B. coli* being retained. Details are given in Appendix IV.

So it seems that a sand filter bed coated with precipitated aluminium hydrate gives a high purification of infected chalk waters until the surface is broken by the movement of algoid growths. But even after considerable growth of algae the purification remains fairly good—better, at any rate, than that given by beds untreated with aluminium sulphate. Probably some of the aluminium hydrate is carried down into the interstices of the sand, where it is unaffected by the surface disturbances caused by the algae.

#### *Filtration through a layer of fine sand.*

Having determined the efficacy of precipitated aluminium hydrate as a filtering medium, I next proceeded to decide whether or not a layer of fine sand on the surface of the filter bed would give equally good results. Some sand was obtained, of which 95 per cent. would pass through a "70 x 70 sieve." One of the filter beds was cleaned, and the fine sand applied an inch thick over the surface. The bed was filled slowly from below, and, after it had been working for a few days, it was infected as before with a peptone water culture of *B. coli*. Hourly samples were taken of the water before and after filtration. The percentage purification was found to be 93·26 %. (For details see Appendix V.) This is better purification than is given by a clean bed without the addition of the inch of fine sand (Table E); but not so good as is given by a recently cleaned bed that has been treated with aluminium

sulphate (Table G). Possibly, by using a foot or more of this fine sand, a high degree of purification could be obtained : but I do not think the method is economical. The cost of the sand, and of the labour needed in washing it, the loss on washing, and the difficulty of manipulating this fine sand, make the use of it as a filtering medium much more costly than aluminium sulphate. At the same time, it is not so reliable. If sand filters are used for the purification of chalk waters, they should be made effective by the precipitation of aluminium sulphate added to the water, rather than by a coating of some fine sand.

*The sterilisation of sand filters.*

The filter beds add certain organisms to the water, as well as abstracting some of the entering organisms (Table D). In order to prevent this addition of organisms to the water, I attempted to sterilise one of the filter beds by the addition of bleaching powder after the bed had been cleaned. At first I added bleaching powder, equivalent to five parts of chlorine per million gallons, and filled the bed from below to the level of the top of the sand with this solution. The bed smelt strongly of chlorine. After a preliminary emptying and washing, the bed was filled in the usual manner and samples of the filtrate taken. The bacterial count was largely increased. Another attempt at sterilisation was made some days later, and 10 parts of available chlorine per million gallons were used. In both cases the solution of bleaching powder was left on the bed for 48 hours. In the second case also the bacterial count was largely increased.

TABLE H.

	Average before adding bleaching powder	Average after adding five parts of chlorine per million gallons	Average after adding ten parts of chlorine per million gallons
Colonies on gelatin in 72 hrs.	55·6 per c.c.	515 per c.c.	345·7 per c.c.
Gelatin liquefiers	26·5 ,,	133 ,,	62·0 ,,

The only explanation that I can offer for the failure of the chlorine to disinfect the deep layers of the bed, is that, throughout the interstices of the gravel and sand, there is organic matter which combines with the free chlorine. The result is that this organic matter is decomposed, and the contained micro-organisms set free to enter the filtrate. The explanation is not wholly satisfactory; but I wish to put on record the paradox observed—namely, that the attempts at sterilisation of a sand filter by means of chlorine made the bacteriological condition of the filtrate no better, but rather the worse.

*Methods of checking the growth of algae in filter beds.*

In the earlier part of this paper, I referred to the trouble caused by the growth and decomposition of algae in the filter beds, and remarked how they gave rise to taste and odour in the water, thereby making it necessary to clean the beds at frequent intervals. I have shown also how the growth and movement of the algae interfered with the high purification in the bed treated with precipitated aluminium hydrate. The algaoid growths are altogether an evil in the sand filtration of chalk waters.

Before I began my work in January, the owners of the water supply had tried the effect of dilute solutions of copper sulphate, applied to the beds during the process of cleaning. No good results had been obtained: on filling the beds subsequently, no inhibition of the growth of algae was observed. Copper sulphate has never been added to the beds while they were working: it is a substance that is undeniably poisonous, and, despite the assertion that has been made, that it does not pass through the sand into the filtrate, I have been unwilling to add it even in small quantities to the water supply.

To one of the beds which was overgrown with weed, I added chlorine derived from bleaching powder in the amount of 5 parts of chlorine per million gallons. This addition was continued daily. In ten days time all the weed was dead, and in three weeks it had decomposed and made the water taste offensively. It was necessary then to clean the filter bed. There was no doubt that the addition of bleaching powder killed the weed.

The next point to determine was whether or not the intermittent addition of bleaching powder to a cleaned filter bed would inhibit the algaoid growths. Two of the beds having been cleaned, bleaching powder was added to each twice a week in the evening, just before the beds stopped working for the day. Five pounds of bleaching powder were thus put into the 250,000 gallons of water in each bed, and the chlorined water left in contact with the filter for twelve hours. No inhibition of the growth of algae was evident, although the addition of bleaching powder was continued during three weeks.

*Covered filter beds.*

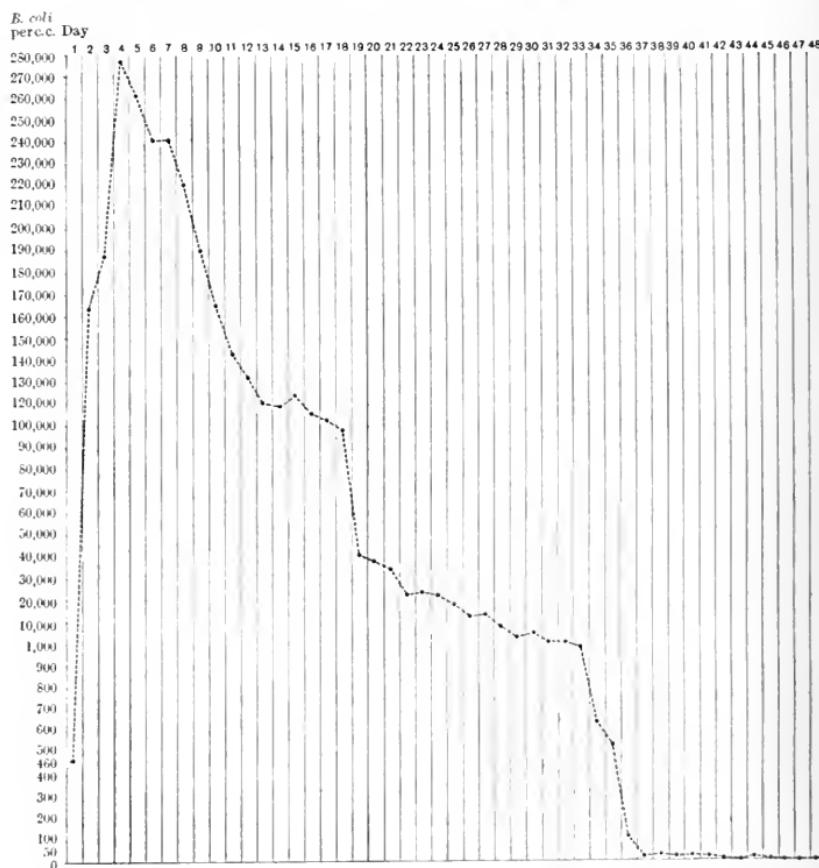
As I have stated, there is a reservoir of five million gallons' capacity into which the filtered water from the three beds is pumped. This reservoir is covered, no growth of algae takes place, nor does the reservoir need to be cleaned; indeed, on such occasions as it has been entered for purposes of cleaning, it has been found that such cleaning was not required. This reservoir has been in use for more than twenty years.

It seems a justifiable inference to make, that if the beds used for filtering a chalk water were covered, there would be no growth of algae, and consequently no need to clean the beds. The cost of covering is about one-third of the original cost of the beds, or about £3000 an acre. Cleaning an acre of filter beds costs roughly about £300 a year. In ten years' time, therefore, the cost of covering would be saved, and, in each subsequent year, the covering would represent a profit. I see no reason why there should be any need for cleaning a covered filter bed, used in the filtration of a chalk water that is only occasionally contaminated.

*The storage of chalk waters.*

Chalk waters do not store well. The few micro-organisms present in the water increase rapidly during the first few days of storage, and this increase, after it has reached the maximum, subsides slowly. Miquel, Frankland and Cramer have all found this increase to obtain during the storage of pure water; and their findings are in opposition to the classical results of Houston, who worked on waters which were ordinarily contaminated heavily. The following curve, based on an experiment of my own, shows the increase and decline in the number of micro-organisms in a chalk water on storage: the water was heavily infected with *B. coli*, and kept in the dark at room temperature.

Storage, then, is not to be recommended for a chalk water, which, on most days in the year, is uncontaminated. Should, however, water from a chalk well be found to be heavily contaminated during the majority of days in the year, then storage possibly would be the best method of treatment before filtration. I do not think it likely that there are any chalk wells which show such invariable bacterial contamination as does, for example, raw Thames water; and the conditions under which storage could be recommended for chalk waters must be very rare.

*Diagram, showing the effect of Storage on a Coli infected Chalk Water.**The purification of a chalk water by softening.*

During the four months of my research I have had an opportunity of observing the effects of two softening installations on the purification of a chalk water. Both these installations are on the Porter Clark principle, and differ only in constructional details, the broad principles of addition of lime followed by preliminary sedimentation, and filtration

through canvas bags being the same in each. Together the plants soften 60,000 gallons of water per day of ten hours.

The water softened comes from the filter beds, and is greatly purified by its passage through the softening installations. The following table based on daily averages, shows the purification effected by softening:

TABLE J.

	<i>B. coli</i> per 100 c.c.	Colonies on agar 24 hrs.	Colonies on gelatin 72 hrs.	Gelatin liquefiers
Unsoftened water	0·27	13·6 per c.e.	150 per c.e.	37 per c.e.
Soft water	0·00	3·0 ,,	21 ,,	0·6 ,,

This shows a purification of 86% on the gelatin count, and 98·4% on the liquefying count.

It has been observed, however, in softening installations, that a pure chalk water is not improved bacteriologically by passing through a softening plant, unless the filtering bags of the latter are sterilised frequently by steam. I did not, therefore, consider that the purification of 86 and 98·4%, shown by Table J, was absolute, because some of the organisms counted in the soft water might have been added by the softening process. I decided for this reason to determine how far the softening plants would cleanse a water infected with some foreign organism; and, since *B. coli* had never been found in the soft water, I used that for a test organism. Peptone water cultures were added to the water before softening; and the number of bacilli in the water before and after softening was determined.

Briefly, the results obtained were as follows: the water passing through a newly cleaned plant is purified very considerably; but a greater purification takes place after the plant has been working for a few hours, and the bags have become coated with a thin deposit of calcium carbonate.

Softening plants Nos. 1 and 11 were cleaned, and the precipitated chalk washed from the bags. A *coli* infected water was then passed through them. No. 1 gave a purification of 98·6%; and No. 11 a purification of 98·76%. In four hours' time No. 11 gave 100% purification; and in five hours' time No. 1 also gave 100% purification. Details are given in Appendices VI and VII.

On another occasion Plant 1 was cleaned again and allowed to work for four hours before the culture of *B. coli* was added. No *B. coli* appeared

in the soft water; the plant gave a purification of 100 %. (Details in Appendix VIII.)

On another occasion the water entering the plants was again infected with *B. coli*. No. 1 plant was newly cleaned: No. 11 had not been cleaned for several days. No. 1 gave 98·8 % purification, and No. 11 a purification of 100 %. (See Appendix IX.)

This last experiment was repeated later. The calcium carbonate was washed from the bags of No. 1 plant. No. 11 was not cleaned, and the bags had a coating of calcium carbonate. During the first 3½ hours of running, the newly cleaned plant gave a purification of 99·08 %; during the next hour practically 100 %; and after that an absolute purification of 100 % of the added micro-organisms. Details are given in Appendix X.

Softening by means of the Porter Clark process is an admirable method of sterilising a chalk water.

Moor and Hewlett have shown in a recent paper that softening by sedimentation alone is not reliable in sterilising chalk waters: the intervention of the filtering bags in the Porter Clark process presumably, therefore, effects the certainty of purification which is lacking in the sedimentation process. The bags very quickly become covered with the finely divided deposit of calcium carbonate, which catches any micro-organism not dealt with by the previous sedimentation. The bacilli do not work their way through this coating of chalk into the effluent; and, when once the maximum purification is reached, the figure is maintained until the plant is cleaned again.

Certain precautions must be taken in using the Porter Clark softening process for the sterilisation of chalk waters. In the first place, it is important that the water passing through a plant during the first four hours or so after cleaning should either be run to waste or else pumped back into the sedimentation tanks. Again, it seems likely that if a plant is overworked or "rushed" the bacteriological condition of the soft water will suffer: for this reason, plants should be installed of capacity sufficient to treat the *maximum* (rather than the average) amount of water to be softened. The canvas filtering bags should be sterilised by steam at regular intervals. If these few simple precautions are taken, in using a Porter Clark installation to sterilise a chalk water, there is no reason why a purification of 100 % should not invariably be obtained.

## CONCLUSIONS.

1. Some deep wells in the chalk are liable to intermittent contamination. The water of such wells ought to be purified before distribution.

2. Open submerged sand filter-beds, as ordinarily used, are not suitable for the purification of chalk waters. They do not effect a sufficient purification from micro-organisms, and they favour the growth of algae which make the water taste and smell unpleasantly.

3. A vital layer does not form on a filter-bed used in the filtration of chalk waters.

4. Besides abstracting some organisms from the water, sand filters add other organisms. This addition is increased by attempts to sterilise the sand filter by means of chlorine.

5. A sand filter can be made an effective filtering agent by the deposition of aluminium hydrate on the surface of the sand. The filter, thus treated, remains efficient until the aluminium hydrate is disturbed by the growth and movement of algae.

6. A layer of very fine sand one inch in thickness on the surface of the filter-bed does not make the bed an effective protection against the passage of micro-organisms.

7. The best way of adapting existing filters to the purification of chalk waters is, to cover such beds so as to exclude the light, and to cause aluminium hydrate to be precipitated over the surface of the sand. A bed, so treated, should not need to be cleaned. Possibly it may be found advisable to add aluminium sulphate periodically during the working of the bed.

8. Bleaching powder, added continuously to a filter-bed, kills any algae that may be present. The intermittent addition of small quantities of chlorine does not inhibit the growth of algae in a filter-bed.

9. Storage is not to be recommended for chalk waters, as it causes a large increase in the number of micro-organisms originally present. Probably treatment with hypo-chlorites or ozone, or passage through some mechanical filter, would purify a contaminated chalk water; but I have no personal experience of these methods.

10. Softening by means of the Porter Clark process is a very excellent way of sterilising a contaminated chalk water. If certain

precautions are observed, this softening method removes all the micro-organisms from the water, and it is therefore more effective than filtration through aluminium hydrate and sand. The Porter Clark softening process is to be recommended, rather than sand filtration, for the purification of a contaminated chalk water.

#### APPENDIX I.

##### *The sand filtration of a coli infected chalk water.*

	Time	<i>B. coli</i> in 1 c.c. of unfiltered water	<i>B. coli</i> in 1 c.c. of filtered water
Feb. 24th	4.30 p.m.	0	0
	5.30 , A	10	1
Feb. 25th	7.30 a.m.	24	2
	8.30 ,	56	0*
	9.30 ,	44	5
	10.30 ,	64	5
	11.30 ,	32	8
	12.30 p.m.	31	10
	1.30 ,	26	2
	2.30 ,	40	1
	3.30 ,	46	3
	4.30 ,	52	8
Feb. 26th	5.30 ,	24	7
	9.0 a.m. B	20	5
	11.0 ,	0†	0*
Feb. 27th	11.30 ,	0	2
Average		33.5 per c.c.	3.9 per c.c.

Percentage purification caused by sand filtration = 88.4 per cent.

Notes:—Culture of *B. coli* added during time between A and B.

\* = 10 c.c. tubes of lactose bile salt peptone water showed acid and gas, and on subculture gave *B. coli*.

† = *B. coli* present in 10 c.c.

## APPENDIX II.

*The sand filtration of a coli infected chalk water.*

	Time	<i>B. coli</i> in 1 c.c. unfiltered water	<i>B. coli</i> per 1 c.c. filtered water	10 c.c. filtered water	25 c.c. filtered water
Wednesday	12.0 m. A	14	0	-	-
	1.0 p.m.	42	0	-	-
	2.0 ,,	36	0	-	+
	3.0 ,,	52	0	+	+
	4.0 ,,	30	1	+	+
	5.0 ,,	104	2	+	+
	8.0 a.m.	60	0	+	+
Thursday	9.0 ,,	64	0	+	+
	10.0 ,,	92	2	+	+
	11.0 ,,	84	3	+	+
	12.0 m.	52	2	+	+
	1.0 p.m.	28	2	+	+
	2.0 ,,	26	0	+	+
	3.0 ,,	10	1	+	+
	4.0 ,,	8	3	+	+
	5.0 ,,	16	1	+	+
	8.0 a.m.	12	2	+	+
Friday	9.0 ,,	10	2	+	+
	10.0 ,,, B	12	0	+	+
	11.0 ,,	5	2	+	+
	12.0 m.	4	1	+	+
	2.0 p.m.	1	2	-	+
	4.0 ,,	1	1	+	+
	8.0 a.m.	0	0	+	+
Saturday	10.0 ,,	0	0	-	+
	12.0 m.	0	0	-	-
		—	—	Percent. purification = 96.9	
		40.1	1.28		

Notes :—Culture of *B. coli* added during time between A and B. Averages taken from figures between dotted lines.

+ = Acid and gas in Lactose MacConkey Medium.

- = No change in Lactose MacConkey Medium.

## APPENDIX III.

*The filtration through sand and precipitated aluminium hydrate of a coli infected water.*

	Time	B. coli per 1 e.c. in unfiltered water	10 c.c. unfiltered water	B. coli per 10 e.c. in filtered water	25 c.c. filtered water
Wednesday	11.0 a.m. A	0	-	0	-
	12.0 m.	64	+	0	-
	1.0 p.m.	88	+	0	-
	2.0 ,,	74	+	0	-
	3.0 ,,	98	+	0	+
	4.0 ,,	94	+	0	+
	5.0 ,,	40	+	0	+
	6.0 ,,	148	+	0	+
Thursday	8.0 a.m.	62	+	0	+
	9.0 ,,	88	+	1	+
	10.0 ,,	110	+	0	+
	11.0 ,,	66	+	8	+
	12.0 m.	12	+	14	+
	1.0 p.m.	8	+	10	+
	2.0 ,,	112	+	24	+
	3.0 ,,	42	+	18	+
	4.0 ,,	124	+	14	+
	5.0 ,,	108	+	20	+
	6.0 ,,	88	+	6	+
	8.0 a.m. B	34	+	2	+
Friday	9.0 ,,	22	+	8	+
	10.0 ,,	0	+	16	+
	12.0 m.	0	+	6	+
	2.0 p.m.	0	+	2	+
	4.0 ,,	0	-	2	+
	6.0 ,,	0	-	14	+
	8.0 a.m.	0	-	2	+
Saturday	10.0 ,,	0	-	6	+
	12.0 m.	0	-	2	+
	2.0 p.m.	0	-	0	+
	4.0 ,,	0	-	2	+
	6.0 ,,	0	-	2	+
	8.0 a.m.	0	-	2	+
Sunday	10.0 a.m.	0	-	4	+
	12.0 m.	0	-	6	+
Monday	10.0 a.m.	0	-	10	+
	12.0 m.	0	-	1	+
	2.0 p.m.	0	-	0	+
	4.0 ,,	0	-	0	+
	6.0 ,,	0	-	0	+

APPENDIX III (*continued*).

	Time	<i>B. coli</i> per 1 c.c. in unfiltered water	10 c.c. unfiltered water	<i>B. coli</i> per 10 c.c. in filtered water	25 c.c. filtered water
Tuesday	10.0 a.m.	0	—	2	+
	12.0 m.	0	—	2	+
	2.0 p.m.	0	—	1	+
	4.0 „	0	—	0	+
	6.0 „	0	—	1	+
Wednesday	8.0 a.m.	0	—	0	+
	10.0 „	0	—	2	+
	12.0 m.	0	—	2	+
	2.0 p.m.	0	—	0	+
	4.0 „	0	—	1	+
Thursday	6.0 „	0	—	0	+
	8.0 a.m.	0	—	2	+
	10.0 „	0	—	0	+
	12.0 m.	0	—	2	+
	2.0 p.m.	0	—	1	+
Friday	4.0 „	0	—	0	+
	6.0 „	0	—	0	+
	8.0 a.m.	0	—	0	+
	10.0 „	0	—	0	—
	12.0 m.	0	—	0	—
	Average per 1 c.c. = 73·9.		Average per 1 c.c. = 0·4.		
	Percentage purification = 99·46.				

*Notes* :—The filtered water was examined in quantities of 10 c.c. and 25 c.c. as indicated. The 10 c.c. samples were plated in Rebipelagar — 2·5 c.c. being added to each of four Petri dishes and about 20 c.c. of the Rebipelagar added to each. For the examination of the 10 c.c. of unfiltered water and the 25 c.c. of filtered water, Lactose MacConkey tubes were used.

The averages were taken from the figures between the dotted lines.

The culture of *B. coli* was added during the time between A and B.

+ = Acid and gas in Lactose MacConkey Broth.

— = No change in Lactose MacConkey Broth.

## APPENDIX IV.

*The filtration of a coli infected water through sand and aluminium hydrate, after there had been much growth of algae in the filter-bed.*

	Time	B. coli per 1 c.c. of unfiltered water	B. coli per 10 c.c. of filtered water	25 c.c. filtered water
Wednesday	11.0 a.m. A	0	0	-
	12.0 m.	12	1	+
	1.0 p.m.	44	0	+
	2.0 ,,	22	4	+
	3.0 ,,	30	12	+
	4.0 ,,	34	12	+
	5.0 ,,	38	16	+
	6.0 ,,	24	20	+
Thursday	8.0 a.m.	22	16	+
	9.0 ,,	38	4	+
	10.0 ,,	42	8	+
	11.0 ,,	30	16	+
	12.0 m.	54	8	+
	1.0 p.m.	60	8	+
	2.0 ,,, B	12	13	+
	3.0 ,,	0	16	+
	4.0 ,,	2	8	+
	5.0 ,,	0	5	+
Friday	6.0 ,,	0	3	+
	8.0 a.m.	1	2	+
	9.0 ,,	0	0	+
	10.0 ,,	0	0	+
	12.0 m.	0	0	-

Average = 33 per 1 c.c.

Average = 0.78 per 1 c.c.

Percentage purification = 97.64.

For Notes see Appendix III.

## APPENDIX V.

*The filtration of a coli infected water through a filter-bed, on the surface of which was an inch of fine sand.*

	Time	<i>E. coli</i> per 1 c.c. of unfiltered water	<i>B. coli</i> per 10 c.c. of filtered water	25 c.c. of filtered water
Friday	10.0 a.m. A	0	0	-
	11.0 ,,	9	0	-
	12.0 m.	10	0	-
	1.0 p.m.	11	0	-
	2.0 ,,	39	10	+
	3.0 ,,	13	0	+
	4.0 ,,	28	15	+
	5.0 ,,	10	20	+
	6.0 ,,	9	5	+
	8.0 a.m.	17	25	+
	9.0 ,,, B	14	15	+
	10.0 ,,	4	8	+
Saturday	11.0 ,,	0	0	+
	12.0 m.	0	0	+
	2.0 p.m.	0	0	-
	4.0 ,,	0	0	-
	6.0 ,,	0	0	-

Average = 14·54 per 1 c.c.

Average = 0·98 per 1 c.c.

Percentage purification = 93·26.

For Notes see Appendix III.

## APPENDIX VI.

*The purification by softening of a coli infected chalk water.  
(Plant No. 1.)*

Time	B. coli per 1 c.c. of unsifted water	B. coli per 10 c.c. soft water	25 c.c. of soft water
11.0 a.m. A	25	0	-
11.15 "	43	0	+
11.30 "	13	3	+
11.45 "	4	2	+
12.0 m.	5	0	+
12.30 p.m.	8	4	+
1.0 "	9	4	+
1.30 "	6	1	+
2.0 "	8	1	+
2.30 "	12	4	+
3.0 "	10	1	+
3.30 "	20	3	+
4.0 "	19	1	+
4.30 "	12	2	+
5.0 "	15	0	+
5.30 "	14	0	-
6.0 " B	15	0	-

Average = 14 per 1 c.c.

Average = 0.2 per c.c.

Percentage purification = 98.6.

*Note:—The Plant (No. 1) was cleaned before the experiment.*

## APPENDIX VII.

*The purification by softening of a coli infected water. (Plant No. 2.)*

Time	B. coli per 1 c.c. of unsifted water	B. coli per 10 c.c. of softened water	25 c.c. of softened water
Tuesday			
11.30 a.m. A	24	0	-
12.0 m.	36	0	-
12.30 p.m.	39	2	+
1.0 "	43	3	+
1.30 "	27	5	+
2.0 "	44	10	+
3.0 "	29	2	+
4.0 "	36	0	-
5.0 "	41	0	-
Wednesday			
8.0 a.m.	10	0	-
9.0 "	2	0	-
10.0 "	0	0	-
11.0 "	1	0	-
12.0 m.	0	0	-

Average = 35.44 per 1 c.c.

Average = 0.44 per 1 c.c.

Percentage purification = 98.76.

*Note:—It will be seen from the above Table and from Appendix VI, that the purification given by these softening plants reaches 100 per cent. after the plants have been working a few hours. This plant was cleaned before the experiment.*

## APPENDIX VIII.

*The purification of a coli infected chalk water by softening.  
(Plant No. 1.)*

Time	B. coli per 1 c.c. of unsoftened water	B. coli per 10 c.c. of softened water	25 c.c. of soft water
11.0 a.m. A	16	0	—
11.15 "	48	0	—
11.30 "	44	0	—
11.45 "	42	0	—
12.0 m.	42	0	—
12.30 p.m.	50	0	—
1.0 "	34	0	—
1.30 "	38	0	—
2.0 "	44	0	—
2.30 "	28	0	—
3.0 "	14	0	—
3.30 "	36	0	—
4.0 "	34	0	—
4.30 "	34	0	—
5.0 "	24	0	—
5.30 " B	20	0	—

Percentage purification = 100.

*Note* :—The plant was cleaned, and then allowed to work for four hours before the culture of *B. coli* was added. During that time the canvas filtering bags became coated with a thin film of calcium carbonate.

## APPENDIX IX.

*The purification by softening of a coli infected water. (Plants Nos. 1 & 2.)*

Time	B. coli per 1 c.c. in unsoftened water	No. 1 Plant B. coli 10 c.c. soft water	No. 2 Plant B. coli 10 c.c. soft water	No. 2 Plant 25 c.c.
10.30 a.m. A	26	0	0	—
11.0 "	190	12	0	—
11.30 "	271	20	0	—
12.0 m.	243	30	0	—
12.30 p.m.	150	20	0	—
1.0 "	149	30	0	—
1.30 " B	151	8	0	—

Average = 169 per 1 c.c.

Average = 2 per c.c.

Percentage purification for No. 1 Plant = 98.8.

" " " 2 " = 100.

*Notes* :—No. 1 Plant was newly cleaned. No. 2 had not been cleaned for several days.

Owing to a mistake, no samples were taken later on the day of this experiment. The Table, however, shows well the difference between a newly cleaned plant and one on the bags of which there is a precipitate of calcium carbonate.

## APPENDIX X.

*The purification by softening of a coli infected water. (Plants Nos. 1 & 2.)*

	Time	<i>E. coli</i> per 1 c.c. of un- softened water	No. 1 Plant.		No. 2 Plant.	
			<i>E. coli</i> per 10 c.c. soft water	25 c.c. soft water	<i>E. coli</i> per 10 c.c. soft water	25 c.c. soft water
Tuesday	11.0 a.m. A	0	0	-	0	-
	11.30 ,,	176	4	+	0	-
	12.0 m.	152	12	+	0	-
	12.30 p.m.	142	20	+	0	-
	1.0 ,,	196	16	+	0	-
	1.30 ,,	180	44	+	0	-
	2.0 ,,	180	20	+	0	-
	2.30 ,,	182	20	+	0	-
	3.0 ,,	160	12	+	0	-
	3.30 ,,	154	0	+	0	-
	4.0 ,,	150	4	+	0	-
	4.30 ,,	152	0	+	0	-
	5.0 ,, B	154	0	-	0	-
Wednesday	8.0 a.m.	11	0	-	0	-
	9.0 ,,	1	0	-	0	-
	10.0 ,,	0	0	-	0	-
	11.0 ,,	1	0	-	0	-
	12.0 m.	0	0	-	0	-
	1.0 p.m.	0	0	-	0	-
	2.0 ,,	0	0	-	0	-
	3.0 ,,	0	0	-	0	-
	4.0 ,,	0	0	-	0	-
	5.0 ,,	0	0	-	0	-
	6.0 ,,	0	0	-	0	-

Average = 164.83 per 1 c.c.

Average = 1.52 per 1 c.c.

Percentage purification given by No. 1 Plant = 99.08.

" " " " 2 " = 100.

*Notes:*—No. 1 Plant was cleaned before the experiment. No. 2 had not been cleaned for several days. It is seen from the figures that when once *B. coli* has been eliminated from the water, it does not reappear—in other words that when the softening plants become efficient to the fullest extent they remain so until the layer of calcium carbonate on the filtering bags is disturbed at the next cleaning.

*For other notes see Appendix III.*

SUPPURATIVE CHOLECYSTITIS WITH CHOLELITHIASIS  
IN A HUMAN "CARRIER" OF THE *BACILLUS  
ENTERITIDIS* OF GAERTNER.

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CHOLECYSTITIS and cholelithiasis are of themselves conditions of great interest from the surgical and pathological standpoint, but the comparatively recent demonstration of the relationship existing between them and the "carrier" state in typhoid fever has made them subjects especially worthy of further study.

In the course of a number of examinations of normal and pathological gall bladders the following observations were made, and seem for several reasons to be of sufficient importance to be put on record. Before entering on these I shall discuss briefly certain questions which have a bearing on the present case.

In spite of the fact that the gall bladder is in communication by means of the bile ducts with the alimentary canal which contains numerous micro-organisms, the bile, under normal conditions, remains sterile, not because it possesses bactericidal properties, but simply on account of its movement towards the gut.

Two views are held as to the paths by which the infective agent finds its way into the gall bladder. The one, whose chief supporter is Chiari, is that where the downward movement of the bile is interfered with by any cause, bacilli ascending the ducts find their way into the gall bladder where they multiply, and, in the case of pathogenic organisms, give rise to pathological changes. The other is that the bacilli find their way into the gall bladder from the blood stream. This view, in favour of a haematogenous origin, has many supporters, and evidence has been adduced in its favour from the appearances found in infection of the gall bladder in man by Joseph Koch (1908) and from experimental

results obtained in animals by Dörr (1905), Chiarolanga (1908), and others. The present case, in regard to this point, affords no evidence in favour of one view more than the other.

In regard to the micro-organisms which have been found associated with cholecystitis and cholelithiasis the most important is *Bacillus typhosus*. Round this subject there has accumulated a great mass of literature which has been recently reviewed by Ledingham (1910).

The organism most frequently present in such cases is the *B. coli communis*. Laubenheimer (1908) out of 36 cases where operation had been performed for empyema of the gall bladder found it 21 times (58·3 %); 18 times in pure cultures (50%). The same observer found among the 36 cases *B. pyocyanus*, *Streptococci*, *Staphylococci*, *B. influenzae*, "Capsule" bacillus and *B. typhosus*. To these must be added the *Pneumococcus* (Etienne) and certain anaerobes (Gilbert and Lippmann (1907)). It is possible that the paracolon bacilli of some of the earlier workers on this subject belonged to the paratyphoid or Gaertner group.

Blumenthal (1907) holds that the *B. coli communis* is probably a secondary invader, the original cause of the condition having died out. He believes that certain organisms of the *coli* group which may have a causal relation to the condition are not identical with the *B. coli communis* of the gut. This view is combatted by Laubenheimer who examined 11 strains of the colon bacillus from cholecystitis and found that in cultural and biological characters they were identical with the *B. coli communis* of the gut.

In regard to cholecystitis due to the paratyphoid bacilli, the group of bacilli most closely allied to the organism found in the present instance, only a few cases are on record: Blumenthal (1904) isolated *B. paratyphosus A* from the material obtained from the gall bladder of a female patient who had been operated on for cholelithiasis. In this case the wall of the gall bladder was thickened and contained gall stones and a small quantity of bile but no pus. Blumenthal found the same bacillus in the stools on one occasion, and the serum of the patient agglutinated the bacillus in a dilution of 1 in 300.

Forster, J., and Kayser, H. (1905) examined post-mortem the contents of the gall bladder in 148 cases. Eight of these had died from typhoid fever, and in seven typhoid bacilli were found. In the eighth case, in which the body had been dead a long time, only *B. coli communis* was found; but typhoid bacilli were found in the blood of a niece who had acted as nurse to the case. Among 140 cases which had died from

diseases other than typhoid fever were two individuals the subjects of cholelithiasis in whom no history of typhoid fever could be ascertained. In the one, aged 84, *B. typhosus* was present, and in the other, aged 71, the *B. paratyphosus* of the type "B." In this case the gall bladder contained abundance of brown bile and two gall stones.

Gaehtgens (1907) found *B. paratyphosus* B in the stools of two female patients who had the symptoms of cholelithiasis, and Dudgeon (1908) found the same organism in a case of suppurative cholecystitis.

Evers and Mühlens (1909) reported an acute case of suppurative cholecystitis with commencing peritonitis following an acute illness lasting three weeks, which was characterised by gastro-intestinal symptoms and the presence of foul-smelling stools. After the operation slimy purulent fluid escaped, and later numerous faceted gall stones. From the gall bladder contents *B. paratyphosus* B was isolated, identified and tested for virulence by Lentz who found it highly virulent. The fluid from the gall bladder gave positive cultural results on several occasions, and the urine and stools were found to contain the same bacillus. Cultures of the bacillus were obtained from the biliary fistula two-and-a-half months after the operation. The blood serum agglutinated the paratyphoid bacillus in a dilution of 1 in 400 and was negative to the typhoid bacillus to 1 in 25.

Hamilton (1910) from 24 cases of cholecystitis isolated 12 organisms, in two cases *B. typhosus*, in three cases *B. coli* and in seven cases *B. paratyphosus* of which four appeared to belong to the "B" and three to the "A" type.

With regard to the association of the *B. enteritidis* of Gaertner with cholecystitis, as in the case about to be recorded, I have not been able to find a single case in the literature available to me. It seems to me that this may be due, not so much to the rarity of the condition, as to the absence in a number of cases of sufficient tests having been applied to enable the determination of the nature of the organism.

In regard to the formation of gall stones it is probable that in the majority of cases, perhaps in all, a bacillary infection is the primary cause. The evidence in favour of this view has been derived not only from observations on operative and post-mortem material from the human subject, but also from experimental results obtained in animals.

Typhoid and paratyphoid bacilli have been found within the gall stones in a number of cases, e.g. Blumenthal (1904-1907), Forster and Kayser (1905) and others. In the case to be recorded *B. enteritidis* Gaertner was cultivated from the gall stones.

As pointed out by Forster the mere finding of the bacilli within the gall stones does not prove that they have a causal connection with the gall stones, for their presence might be interpreted in another way, viz. that they had grown into the gall stone after its formation; indeed Gilbert and Baemeister have proved experimentally (the former in porous cholesterine stones, the latter in calcium-cholesterine stones) that secondary invasion of the calculi can take place. Aschoff and Baemeister have, moreover, several times found typhoid bacilli in the centre of large gall stones in cases of typhoid fever in the second or third week. The writer had recently in his laboratory a similar experience. They maintain that the bacilli must have wandered into the already formed calculi. No evidence, however, is adduced that these were not second attacks, the bacilli having persisted in the gall bladder and calculi from an earlier infection.

The formation of calculi with an accompanying first attack of biliary colic soon after an attack of typhoid fever is strongly in favour of the view that the gall stones are of bacillary origin. A number of such cases have been recorded. Hilgermann (1909) reports a case where calculi were found within two months of the recovery from typhoid fever. The results obtained by animal experiments strongly support the bacillary origin of the calculi. As early as 1899 Richardson found that an injection of typhoid bacilli into the gall bladder of a rabbit was followed by the formation of calculus, and Cushing (1899), Dörr (1905) and others obtained like results from the same animals by intravenous injection of typhoid bacilli.

It is unnecessary to refer to the extensive literature on this subject. It is sufficient here to say that evidence will be adduced in this paper which appears to indicate that the *B. enteritidis* Gaertner stands in relation to the production of cholecystitis and cholelithiasis on the same footing as the typhoid and paratyphoid bacilli.

#### *The present investigation.*

*History of the case.* Case of Mrs M. C. aged 61. No previous illness except inflammation of the lungs when young. There was no history of acute food poisoning. The patient when 22 years of age had "gastric fever." Before her present illness she had frequent attacks of indigestion. The present illness commenced about ten years ago with pain in the region of the gall bladder. The pain came in spasms, and was very often severe, shooting through to the back and up to the right shoulder.

It lasted about a fortnight at first, then went away and recurred again and has kept on recurring at intervals till five years ago when it ceased and at the same time she passed a calculus about the size of a hazel nut. For the next four years she had no trouble at all. About one year ago the pain again commenced to trouble her, and every time it was severe she became of a yellow colour which went after cessation of the pain. This occurred several times during the year.

*Physical examination.* On inspection of abdomen nothing unusual to be seen. On palpation there is distinct tenderness and resistance over the gall bladder region, which is worse when deep palpation is performed, especially during inspiration. There is no enlargement of the gall bladder to be felt, and no tumour. Liver extends to about half-an-inch below the costal margin.

She was admitted to the Aberdeen Royal Infirmary on November 3rd, and was operated upon on the 5th, cholecystotomy being performed, and the patient was discharged on the 21st. On February 9th she had an attack of pain over the left lobe of the liver and on the following day had marked jaundice of the skin and conjunctiva, and bile was demonstrated in the urine.

I am indebted to Dr Fowler, Woodside, and to Mr H. M. Gray, Aberdeen, for the above history, to the former for samples of blood and faeces and to the latter for the pus, gall stones, and part of the gall bladder.

*Examination of part of the wall of the gall bladder.* The fundus, which alone was available for examination, was considerably thickened, reaching from 5 to 7 mm. The mucous membrane was folded and covered with an admixture of blood, pus and bile.

*Histological examination.* The gall bladder showed, in addition to the folding of the mucous membrane, a new formation of epithelial bud-like processes. The vessels in all the layers were dilated, and this was especially marked in the mucous membrane. The vascular dilatation was accompanied by numerous haemorrhages, some large enough to be visible to the naked eye on section. There was present a round-cell infiltration of all the coats which was most pronounced in the mucous membrane, submucosa and muscularis. The cells consisted chiefly of lymphocytes and fibroblasts, but a few polymorphs were present. There was some fibrous thickening of the serous coat. The appearances, therefore, are those associated with recurrent or chronic chole cystitis. Gram-negative bacilli were demonstrated in the wall by Unna's polychrome-methylene-blue method.

*Bacterial examination.* With the fundus of the gall bladder removed during the operation, some of the contained fluid and six gall stones were received for examination. Some of the fluid (a mixture of bile, pus and blood) was plated on MacConkey's neutral-red bile-salt agar; only non-lactose fermenting colonies appeared. The gall stones were about the size of large peas, and were of the calcium-cholesterine type. One of these was dipped in methylated spirit, which was burned off, and the calculus was then ground up with some broth in a sterile mortar. The mixture was plated as before and gave the same result.

Samples of faeces taken a week after the operation, and three months after the operation, gave non-lactose fermenting colonies. The non-lactose fermenting organisms obtained from these four sources were submitted to further examination and appeared to be identical.

#### *Characters of the bacillus isolated.*

The bacillus was a Gram-negative rod, slightly motile, did not liquefy gelatin and did not produce indole. It gave the following fermentation reactions: acid and gas were produced on glucose, mannite, dulcite, maltose, mannose, galactose, arabinose, sorbite, whereas no marked change took place in lactose, saccharose, raffinose, inulin, dextrin, salicin, erythrite.

Litmus milk became slightly acid in the 24 hours, then alkaline, and in 15 days this alkalinity was very pronounced. From these reactions, therefore, the organisms appear to belong to the group comprising *B. suis*, *B. paratyphosus* B, *B. enteritidis* Gaertner, *B. typhi murium*, *B. aertryck*, and at first it was suspected that one had here a carrier of the *B. paratyphosus* B, as several such cases had already been recorded.

A study of the pathogenicity of the bacillus, further agglutination and absorption tests, and a comparison with well authenticated strains proved, however, that the bacillus was the *B. enteritidis* Gaertner from the original strain of which it was indistinguishable.

*Pathogenic effects.* Two guinea-pigs were inoculated intraperitoneally with the bacillus. One received 250 mgrm. of a broth culture (24 hours) and died within 48 hours of acute peritonitis and general infection. The second animal received 10 mgrm. and died in 10 days. In this case there was a milky deposit on the peritoneum, pale yellow or almost white nodules about the size of millet to hemp seed in the liver and in the spleen which was enlarged. From both animals the bacillus was isolated in pure culture. *Rabbits.* One of these died during immuni-

sation and presented post-mortem appearances closely resembling those described in the second guinea-pig. *Rats.* Three of four young rats, which were fed with cultures of the bacillus, died with the symptoms and post-mortem signs of acute enteritis.

### *Agglutination tests.*

I was fortunate in possessing a number of sera for which I was indebted to Dr Bainbridge, enabling me at once to carry out the primary agglutination tests. The results of agglutination tests with these sera correspond to the various members of the group shown on Table I.

TABLE I.

Serum	Pus	Gall stone B.	Faeces B.
<i>B. paratyphosus A</i> (titre 20,000)	< 100	< 100	< 100
<i>B. paratyphosus B</i> (titre 2000)	< 100	< 100	< 100
Also for <i>B. aertryck</i> , 1000			
<i>B. typhi murium</i> (titre 4000)	< 100	< 100	< 100
<i>B. aertryck</i> (titre 5000)	< 100	< 100	< 100
<i>B. Gaertner</i> (titre 8000)	6400	6400	6400

All the agglutination tests were carried out by the macroscopic method in small test tubes.

C = Complete sedimentation.

++ = Marked agglutination.

+ = Slight agglutination.

TABLE II.

### Serum (Faeces Bacillus).

Bacillus	Dilution...100	200	400	800	1600	3200	6400	Control
<i>B. Gaertner</i> (original)	C	++	++	+	+	0	0	...
Faeces B. ...	C	++	++	+	+	0	0	...

### Serum (Pus Bacillus 16, 1, 11).

<i>B. Gaertner</i> (original)	C	C	C	C	++	0	0	0
Gall-stone B. ...	C	C	C	C	++	+	0	0
B. from faeces 23/1/11	C	C	C	C	++	+	+	0
Pus B. ...	C	C	C	C	++	+	+	...

### Serum (Gall-stone Bacillus).

<i>B. Gaertner</i> (original)	C	C	C	++	0	0	0	...
Gall-stone B. ...	C	C	C	++	+	0	0	...
Pus B. ...	C	C	C	++	0	0	0	...
Faeces B. ...	C	C	C	++	0	0	...	...

The serum of the patient was also tested against its own bacilli and against the original strain of Gaertner, and in both cases gave distinct agglutination in a dilution of 1-40.

Rabbits were immunised with three of the races of bacilli isolated from the case and a number of the agglutination tests were carried out with these. Some of the results of these tests, at different stages of agglutination, are shown on Tables II, III, IV.

The result of an absorption test is shown on Table V.

TABLE III.

Serum (Gall-stone Bacillus).							
Bacillus	Dilution...1000	20000	40000	80000	160000	Control	
Gall-stone B.	...	++	+	0	0	0	...
Faeces B.	...	++	+	0	0	0	...
Pus B.	...	++	+	+	0	0	...
<i>B. Gaertner</i>	...	++	+	0	0	0	...

Serum (Pus Bacillus).							
Gall-stone B.	...	++	++	0	0	0	0
Faeces B.	...	++	+	0	0	0	0
Pus B.	...	++	++	+	0	0	0
<i>B. Gaertner</i> (original)	...	++	+	0	0	0	0

TABLE IV.

*Agglutination of Bacilli isolated from Faeces on 25/2/11, i.e. about three months after operation.*

Gaertner Serum (titre 8000).							
Dilution...100	200	400	800	1600	3200	6400	Control
C	C	C	C	C	++	+	0

TABLE V.

*Agglutination at dilution of 1-80 after absorption.*

Serum	Absorbed by	<i>B. Gaertner</i>	Pus B.	Gall-stone B.	Faeces B.
<i>B. Gaertner</i>	0	8000	6400	6400	6400
<i>B. Gaertner</i>	0	0	0	0	0
Gall-stone B.	0	0	0	0	0

## DISCUSSION.

The *B. enteritidis* Gaertner, the organism with which we are dealing, is a member of the group of bacilli associated with the production either of outbreaks of acute food poisoning, where the dose or the virulence of the infective material has been great, or of a less acute typhoid-like gastro-enteritis where the dose has been small, the virulence low, or the resistance great.

The other more important members of the group are *B. paratyphosus* B, *B. suis*, and *B. aertryck*, the two latter being regarded by many authorities as identical.

The outbreaks of food poisoning due to *B. enteritidis* Gaertner have usually followed the ingestion, in some form or another, of the flesh of the horse, ox or pig, and not infrequently it has been found that the animals from which the material originated had been in a diseased condition at the time of slaughter. This, however, has not been invariably the case and the observations here recorded are of interest from the standpoint that they may help to explain how in certain cases sound meat may become contaminated by human handling.

Some scepticism has been shown by certain workers as to the pathogenic importance of this group of bacilli. They claim to have found them in non-toxic food stuffs and also in healthy individuals, and as a result show a tendency to regard them as ubiquitous. If this view were correct, the observations in this communication would be deprived of their chief significance. This sceptical view, however, is not borne out by the work of Hilgermann (1910) who holds that the majority of cases of infection due to *B. paratyphosus* B. can be traced to infected individuals. The work of Otto Meyer (1909) is also opposed to the view, for out of a large number of examinations he found *B. paratyphosus* B. only twice in the stools of healthy individuals and both proved to be contacts; the one with an "acute" and the other with a "carrier" case. In the stools of over a hundred healthy soldiers he did not find it once.

The *B. enteritidis* Gaertner has been proved to be a cause of certain epizootics among rabbits, guinea-pigs and rats. This may have some significance in relation to human infection in view of the widespread use of rat viruses which contain this bacillus (Bainbridge (1909)). Apart from these, the bacillus is by no means widespread among human beings or the lower animals, and endeavours to find it in the

discharges, etc. of normal man and animals have almost invariably failed.

There is much evidence confirming the earlier observations in regard to *B. enteritidis* Gaertner made by van Ermengem who failed, out of a large number of examinations, to find the bacillus in the stools of healthy individuals, in the organs from bodies advanced in putrefaction, in putrefying food stuffs, etc. Among recent evidence in this direction may be quoted the work of Aumann (1911), working under Dunbar and Trautmann, who, after an elaborate investigation, asserts that the bacilli of the paratyphoid and Gaertner group are not found apart from disease in man or animals. When they occur in healthy animals they must be regarded as "carriers" in the same sense as human "carriers."

Zweifel's (1911) researches led to the same conclusion. The case reported is of interest in this connection for it goes to prove that in certain cases *B. enteritidis* Gaertner may have its habitat in the gall bladder and be shed out periodically in the faeces in a manner exactly analogous to that which occurs in the case of the typhoid and paratyphoid bacilli. In this case there must be the same risks of infection as have been proved to exist in the case of typhoid and paratyphoid.

Infection may be by contact but, as has been shown in those conditions, the danger is greater where the "carrier" is an individual dealing with food stuffs.

#### SUMMARY.

In a case of suppurative cholecystitis with cholelithiasis a bacillus was isolated from the pus, gall bladder, and stools which has the morphological, biological and cultural characters of *B. enteritidis* Gaertner. This bacillus is pathogenic for the rat, rabbit and guinea-pig, and gives rise to the characteristic lesions produced by the Gaertner bacillus. The serum of the patient agglutinated the three strains of bacilli isolated from the above mentioned sources and the original Gaertner strain in a dilution of 1 in 40. A serum, prepared by injecting *B. enteritidis* Gaertner (original strain) into a rabbit, agglutinated the three strains isolated from the case and the homologous bacillus in almost the same dilutions.

A serum prepared from any of the three bacilli agglutinates the homologous organism, the other two strains and the original Gaertner bacillus in almost the same titre. The sera homologous to allied organisms do not agglutinate these bacilli. Absorption tests confirm these results

and prove that the bacilli isolated are identical with the Gaertner bacillus.

The chief points of interest in the case are that :

1. It forms a link in the chain of evidence showing that as in the case of the paratyphoid bacillus the *B. enteritidis* Gaertner may give rise, not only to the more acute toxic form of poisoning, but also to a sub-acute paratyphoid type of the disease.

2. It establishes the association of *B. enteritidis* Gaertner with suppurative cholecystitis and with cholelithiasis.

3. It proves that this bacillus may have its habitat in the gall bladder and be shed out at intervals into the faeces, in a manner exactly analogous to what occurs in the case of typhoid and paratyphoid infections.

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A MEDIUM FOR *BACILLUS DIPHTHERIAE*  
(POTASSIUM-SULPHOCYANIDE NEUTRAL-RED GLUCOSE  
SERUM).

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THE preparation of a medium that would allow the bacillus of diphtheria to grow and exclude all other organisms would be an ideal medium for those whose work is concerned with that organism, but, as such a medium has not yet been devised, we must be content with the modifications of the ordinary media which we possess. Various varieties of media have been prepared for the purpose of favouring the growth of *B. diphtheriae*, but very little seems to have been done with regard to selective media.

Dr Myer Coplans, of Leeds, has been experimenting with numerous varieties of selective media for some time and, of late, I have been using his various media both in their modified and original forms. The following notes are concerned with the results obtained by the use of such media and, as over 6000 cultures were examined in this hospital during the past half-year, the results may be of some value and may influence others likewise to experiment with such media. On an average the number of cultures examined yearly in this institution is between 11,000 and 12,000. Dr Coplans has done most of his work with agar and plates, while I have used mainly blood serum in tubes. Agar tubes have been used in this hospital, but the results were not so satisfactory as those obtained with blood serum. The medium originally used in this institution consisted of three parts of blood serum, one part of bouillon, and glucose 0·5%.

This medium was very good, but it did not present any selective properties. The first modification was the addition of neutral-red. A

0·5% watery solution of neutral-red was prepared and various quantities of this were added. After many experiments it was found that 2% of this solution gave the best results. Solutions of 0·5%: 1%: 1·5%: 2%: 3% and 5% were added and gave more or less satisfaction. The 2% solution, however, gave the best coloration and enabled one to distinguish the varieties in colour produced by the various organisms. The use of neutral-red as an indicator is by no means new and has been used by many bacteriologists. The neutral-red becomes pink in the presence of acid and yellow in the presence of alkalies. *B. diphtheriae* is an acid-producing organism when glucose is present and renders the neutral-red pink, but, unfortunately, this organism is not the only one of those found in the respiratory passages that produces acid. Consequently, the production of acid is not diagnostic of *B. diphtheriae*. The next step in the scheme was to find a substance which would allow the bacillus to grow and inhibit the other acid-producing organisms. Unfortunately, this substance was not found, but it was found possible to inhibit the growth of some of the organisms and render the detection of bacillus of diphtheria more easy. The salts of potassium were added to the medium in rotation e.g. potassium cyanide, potassium sulphocyanide, potassium ferrocyanide, and potassium ferricyanide, in various proportions. The potassium sulphocyanide acted best and the amount introduced was 1%. I have come to the conclusion that this is the best proportion and ought not to be departed from. The introduction of larger or smaller quantities has not been a success and any apparent failures, I have had, have been due to proportions of this salt other than 1%. The medium containing the neutral-red and potassium sulphocyanide has proved to be the best medium. It allows the bacillus to grow and seems to inhibit the growth of many of the other organisms. Cocci grow well and some of them produce a red colour somewhat similar to that produced by *B. diphtheriae*. Experience soon enables one to distinguish the colour produced by the cocci and that produced by the diphtheria bacillus. *B. megatherium* and *B. subtilis* are not uncommon in cultures and it has been found that the above medium inhibits their growth for some hours. It must not be thought that their growth is prevented; such is not the case. The point is that they are inhibited for a sufficient time to enable us to diagnose the presence of *B. diphtheriae*, and this only applies to the number present in ordinary cultures and not to pure cultures of the organisms. Out of the thousands of tubes examined here only in a few cases was the medium contaminated sufficiently to render it useless.

A further series of experiments was conducted with the addition of bile salt to the medium containing neutral-red and potassium salts. Then the combination of various potassium salts with and without bile salt was tried. The results of these experiments were valueless except to indicate that the addition of bile salts was a useless proceeding.

The various constituents of these media are of use for certain specific purposes, but did not further the investigation with regard to diphtheria.

The sum total of all these experiments is that the coagulated and sterilized medium consisting of

Blood serum of the sheep	3 parts
Bouillon	1 part
Glucose	0·5 %
Potassium sulphocyanide	1 %
·5 % sol. (watery) of neutral-red	2 %

is the best medium for the bacillus of diphtheria.

The value of this medium is that it can enable one to say when the bacillus is present or absent without the aid of a microscope. When the bacillus is present a pink colour is produced. A pink colour is produced by cocci, but, by experience, this can be distinguished from that produced by *B. diphtheriae*. When diphtheria is absent there is no pink colour; this is the main point of the medium. One can definitely say when *B. diphtheriae* is absent. This is of importance when many tubes have to be examined as it is possible to dispense with the microscope and save valuable time. I have used over 3000 tubes of this medium and have not had a case in which the non-pink medium showed *B. diphtheriae*. Up to the present, I have not dispensed with the microscope, but have verified the result in every case. Of course, infallibility is not claimed for it, but considering the results I have obtained until now I think it is worth a trial.

There are a few points to be mentioned in conclusion :

- (1) Use good serum. The quality of the serum influences the medium greatly.
- (2) Always add glucose.
- (3) Use 1 % potassium sulphocyanide and make it fresh each time.
- (4) Use 2 % of a 0·5 % watery solution of neutral-red.
- (5) Do not keep the medium too long. I have not had any medium kept for more than six weeks.

## DIFFERENTIAL MEDIA FOR RECOGNITION OF *B. DIPHTHERIAE* AND ASSOCIATED ORGANISMS.

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CERTAIN difficulties attendant upon bacteriological diagnosis recur constantly in connection with the notification, isolation, and discharge of patients suffering from diphtheria, and from every point of view it becomes increasingly necessary to seek means to minimise, as far as possible, the number of 'missed' and 'carrier' cases.

The ready bacterioscopic recognition of certain organisms prone to infect the upper respiratory tract is by no means sure, and, more especially with diphtheria, negative results are often misleading.

Particularly with subcultures of suspected material on media the basis of which is animal blood-serum, failure to recover the diphtheria bacillus is often due to overgrowth of associated organisms: occasionally the difficulty may be explained away by the invocation of latency, in that the transplanted organism does not take kindly at first to its new home. The comparatively rapid growth of *B. subtilis*, *B. megatherium*, *B. Hofmanni*, and of certain *Torulae* or *Micrococci* is the usual source of trouble.

From sowings of an admixture of *B. diphteriae* and *B. subtilis* or *B. megatherium* I have often been able to recover the former from a surface growth on coagulated blood-serum by scraping along the upper dried semi-hornified portion of the serum precisely at that point or margin where the culture of *B. subtilis* or *B. megatherium* ceased to spread: it is a matter of greater difficulty to recover *B. Hofmanni* when present in place of *B. diphteriae* in like mixtures.

Whereas the condition of dryness in a serum culture is unfavourable for the development of *B. subtilis* and *B. megatherium* it favours the growth of *B. diphteriae*, a fact which may, perhaps, be explained by the close relationship of the latter with the myco-bacteria.

## I. AGAR MEDIA.

*Dryness of Medium.* For practical purposes 'dryness' of a medium may be induced by the addition of salts, such as chloride of calcium, or by the addition of substances such as glucose or glycerine.

For convenience in rapid working nutrient agar was used as a basis in place of blood-serum, especially in the earlier series of experiments to be described. Surface streaks on plates were made and the cultures incubated at 37° C. with the following results:—

(1) (a)  $\text{CaCl}_2$  5% added: *B. diphtheriae* and *Staphylococci* plentiful growth; *B. subtilis*, slight growth; *B. Hofmanni*, *B. megatherium* and *B. coli* trace only.

(b)  $\text{CaCl}_2$  2½% added: *B. megatherium* and *B. coli* alone markedly restrained; some restraint of *B. Hofmanni*.

(2) Glucose 10% added: *B. diphtheriae*, *B. subtilis* and *B. Hofmanni* apparently not restrained. Growth of *B. coli* and *B. megatherium* appears to be inhibited.

*Action of Salts of Saliva and Bile.* Neither glucose nor calcium chloride alone or in conjunction would appear to render a medium specially favourable to *B. diphtheriae* while excluding the other organisms already specified. The scope of the experiments was therefore extended so as to determine the action of certain salts of the saliva and bile, potassium sulphocyanide and taurocholate of soda respectively, both in agar and coagulated blood-serum media. In addition, both ammonium and sodium sulphocyanide have been tested, and, as an indicator to determine the reaction of the medium, 1% of a 1% watery-neutral red has usually been added. Fuchsin, decolorised by alkali, has on occasion been tested as an indicator, but there is little advantage attending its use.

(3) The addition of ammonium sulphocyanide to agar medium already containing glucose inhibited the growth of the following organisms in the order:

(a) *B. megatherium*, (b) *B. coli*, (c) *B. diphtheriae*, (d) *B. subtilis*, (e) *B. Hofmanni*, which is least affected, not being, apparently, unrestrained even in presence of 2½% AmCNS.

(4) AmCNS, in the presence of  $\text{CaCl}_2$ , continued to inhibit especially the growth of *B. Hofmanni*.

(5) ½% bile salt, in presence of 5%  $\text{CaCl}_2$ , appeared to inhibit greatly the growth of all the organisms considered.

(6)  $\frac{1}{2}\%$  bile salt, in presence of glucose and ammonium sulphocyanide, favoured especially the growth of *B. Hofmanni*, and, with neutral-red, a purple-red growth results surrounded by a clear zone.

The foregoing series of tests were carried out by way of a preliminary trial. In the following series all the media were composed of nutrient agar to which neutral-red has been added as specified, together with certain salts. The cultures were incubated at  $37^{\circ}\text{C}$ .

(A) *Potassium Sulphocyanide* was added in quantities varying from  $\frac{1}{8}\%$  to  $2\frac{1}{2}\%$ —with the result that  $\frac{1}{4}\%$  and over inhibited the

#### *A Series.*

Colour denotes appearance of colonies : T. L. = transmitted light, R. L. = reflected light.

		<i>B. diph-</i> <i>theriae</i>	<i>B. sub-</i> <i>tilis</i>	<i>B. coli</i>	<i>B. mega-</i> <i>therium</i>	<i>B. Hof-</i> <i>manni</i>	Mixture of <i>Staphy-</i> <i>lococci</i> and <i>B. Hofmanni</i>
Control, neutral red agar plate	T. L.	Faint pink	Salmon pink	Bluish pink	Bluish fluorescence	Bluish pink	Brick red
KCNS omitted	R. L.	Pinkish	Pinkish	Pinkish	Pinkish	Pinkish	Pinkish
Amount of growth		3*	3	3	2	3	3
$\frac{1}{8}\%$ KCNS added	T. L.	Pink	Yellow red	Brick red	Cream	Yellow	Yellow
	R. L.	Claret red	Yellow red	Yellowish	Cream	Yellow	Yellow
	Growth	3	3	2	3	3	3
$\frac{1}{4}\%$ KCNS added	T. L.	Pink	Yellow red	Brick yellow red	Cream	Yellow	Brick yellow
	R. L.	Red	Yellow red	Yellow	Cream	Yellow	Yellow
	Growth	3	3	3	1	3	3
$\frac{1}{2}\%$ KCNS added	T. L.	Pink	Yellow red	Brick yellow red	--	Brick yellow	Yellow
	R. L.	Claret red	Brick	Yellow	--	Yellow	Yellow
	Growth	3	3	3	$\frac{1}{2}$	3	3
$1\%$ KCNS added	T. L.	Claret red	Brick red	Pink	Very faint pink	Yellow red	Yellow red
	R. L.	Claret red	Brownish	Yellow red	--	Yellow red	Yellow red
	Growth	2	3	1	$\frac{1}{4}$	3	3
$2\frac{1}{2}\%$ KCNS added	T. L.	Reddish	Brick	--?	--?	Red	Brick red
	R. L.	Deep pink	Brick	--?	--?	Deep pink	Deep pink
	Growth	2	3	?	?	2	3

\* The numbers  $\frac{1}{2}$ , 1, 2, 3, 4 in the tables indicate progressive degrees of growth measured objectively.

growth of *B. megatherium*, and 1% and over inhibited that of *B. coli*. Where KCNS is present to the amount of  $2\frac{1}{2}\%$  or less, the naked-eye appearance of colonies of *B. diphtheriae* both by transmitted and reflected light was sufficiently distinctive; above  $2\frac{1}{2}\%$  the tints developed by the colonies were not sufficiently distinctive to be of value.

(B) *Bile Salt* (taurocholate of soda-ox-bile) was added in quantities varying from  $\frac{1}{8}\%$  to 5% to neutral-red agar and plates poured. Prolonged incubation at 37°C. Results: with  $\frac{1}{4}\%$  there is some restraint and with  $\frac{1}{2}\%$  complete inhibition of growth of *B. subtilis* and *B. megatherium*. The addition of  $\frac{1}{4}\%$  to 1% shows up special coloration of colonies of *B. diphtheriae* when present in pure culture.

### *B Series.*

Control—see A series.

		<i>B. diph-</i> <i>theriae</i>	<i>B. sub-</i> <i>tilis</i>	<i>B. coli</i>	<i>B. mega-</i> <i>therium</i>	<i>B. Hof-</i> <i>manni</i>	Mixture of <i>Staphy-</i> <i>lococci</i> and <i>B. Hofmanni</i>
$\frac{1}{8}\%$ Bile salt added	T. L.	Yellow	Bluish fluoresc.	Bluish fluoresc.	Bluish fluoresc.	Bluish yellow	Bluish yellow
	R. L.	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
	Growth	3	2	4	2	3	3
$\frac{1}{4}\%$	T. L.	Red yellow	Yellow	Yellowish- blue fluoresc.	Bluish fluoresc.	Bluish Yellow	Yellow
	R. L.	Red	Red	Red	Red	Red	Red
	Growth	3	1	4	Trace	3	3
$\frac{1}{2}\%$	T. L.	Red	—	Yellow fluoresc.	—	Yellow	Reddish pink
	R. L.	Red	—	Yellow fluoresc.	—	Yellow	Reddish pink
	Growth	2	Trace	4	0	4	4
$1\%$	T. L.	Red	—	Yellowish- blue fluoresc.	—	Yellowish blue	Yellowish pink
	R. L.	Red	—	Yellow fluoresc.	—	Yellow	Yellowish pink
	Growth	2	0	4	0	4	3
$5\%$	T. L.	Red	—	Red	—	Yellow centre, red periphery	Red
	R. L.	Almost black	—	Red	—	Yellow centre, red periphery	Red
	Growth	2	0	2	0	3	3

(C) *Calcium Chloride* added to neutral-red agar plates. The effect of increasing the percentage of  $\text{CaCl}_2$  in agar was to restrain the growth of all the organisms which were cultivated upon the

medium. The presence of 1% to  $2\frac{1}{2}\%$  of this salt allowed *B. diphtheriae* and certain *Micrococci* to grow freely while inhibiting markedly the growth of *B. subtilis*, *B. coli*, *B. Hofmanni*, and *B. megatherium*. With 1%  $\text{CaCl}_2$  there appeared a distinct clear zone around the red growth of cocci, while throughout the medium generally there was a faint haze.

*C Series.*

		<i>B. diph-</i> <i>theriae</i>	<i>B. sub-</i> <i>tilis</i>	<i>B. coli</i>	<i>B. mega-</i> <i>therium</i>	<i>B. Hof-</i> <i>manni</i>	Mixture of <i>Staphy-</i> <i>lococci</i> and <i>B. Hofmanni</i>
Control. No $\text{CaCl}_2$ added	R. L.	Pink	Pink	Faint pink	Pink	Pink	Red
	T. L.	Red	Bluish pink	Bluish fluoresc.	Bluish fluoresc.	Bluish fluoresc.	Yellow
	Growth	3	3	3	3	3	4
$1\%$ $\text{CaCl}_2$	R. L.	Faint red	Pink	? growth	Very faint pink	Faint pink	Clear zone arounded growth
	T. L.	Faint red	Pink	? growth	—	—	Ditto
	Growth	2	2	½	¼	2	3 <sup>(a)</sup>
$2\frac{1}{2}\%$ $\text{CaCl}_2$	T. L.	Pink	Pink	—	—	—	Reddish pink
	R. L.	Pink	—	—	—	—	Reddish pink
	Growth	3	1	¼	¼	½	3 <sup>(b)</sup>
$2\frac{1}{2}\%$ $\text{CaCl}_2$ . Another series of strains of organisms used		1	1	0	0	Trace	—
	5% $\text{CaCl}_2$	{ T. L. R. L.	Pink —	Pink —	— —	Pink —	Pink <sup>(c)</sup> —
	Growth	1	1	¼	¼	1	1
7½% $\text{CaCl}_2$	...	Trace	Faint trace	0	0	Trace	Faint trace

(a) 1%  $\text{CaCl}_2$ —growth: nearly all cocci. (b)  $2\frac{1}{2}\%$   $\text{CaCl}_2$ —practically all cocci. (c) 5%  $\text{CaCl}_2$ —all cocci. Thus the addition of  $\text{CaCl}_2$  would appear to inhibit the growth of *B. Hofmanni* in a mixed culture containing *Staphylococci*, *B. Hofmanni* and *B. diphtheriae*, a result which was confirmed by subsequent experiment.

(E), (O) and (I). In addition to potassium sulphocyanide various other cyanides—potassium cyanide (= E Series), potassium ferrocyanide (= O Series), and potassium ferricyanide (= I Series)—were added in varying percentages to neutral-red agar plates. Surface streak cultures were made and incubated as before.

*Tints:* R = Reddish, W = Whitish, Y = Yellow, Br = Brownish.

The numbers  $\frac{1}{2}$ , 1, 2, 3 denote progressive degrees of growth.

*E Series.*

	<i>B. diphtheriae</i>	<i>B. subtilis</i>	<i>B. coli</i>	<i>B. megatherium</i>	<i>B. Hofmanni</i>	Mixture of <i>cocci</i> and <i>B. Hofmanni</i>
KCN % added, $\frac{1}{4}$	R 3	W 3	W 1	R 2	Y 2	Y 3
$\frac{1}{2}$	R 1	W 2	0	P 2	Br 2	Br 3
1	R 3	W 3	W $\frac{1}{2}$	Y 2	Y 2	Y 3
$2\frac{1}{2}$	0	W 3	0	W 3	0	W 3

Much inconvenience is experienced in working with media to which potassium cyanide is added owing to the constant liberation of prussic acid whilst pouring the plate and during period of incubation.

Owing to this continual discharge of HCN vapour the medium tends to become increasingly alkaline and yellow as incubation proceeds, and therefore at the moment of pouring the plates the reaction of the medium should be adjusted with free HCl so as to indicate faint acidity.

In general *B. diphtheriae* gives a reddish coloration which is slightly distinctive and serves to differentiate its colonies from those of the remaining organisms with the exception of *B. megatherium*. The colours fade rapidly changing to pale yellow with the development of increased alkalinity of the medium.

(O) *Potassium Ferrocyanide.*

	<i>B. diphtheriae</i>	<i>B. subtilis</i>	<i>B. coli</i>	<i>B. megatherium</i>	<i>B. Hofmanni</i>	Mixture of <i>Staphylococci</i> and <i>B. Hofmanni</i>
% K <sub>4</sub> FeC <sub>6</sub> added, $\frac{1}{4}$	R 3	W 3	W 2	R 3	YBr 3	R 3
$\frac{1}{2}$	R 3	W 2	0	PR 3	BrR 2	R 3
1	0	W 3	0	PR 3	BrR 3	R 3
$2\frac{1}{2}$	0	W 3	0	W 3	YR 3	R 3

Thus the salt in quantities exceeding  $\frac{1}{4}\%$  serves to inhibit *B. coli*. *B. diphtheriae* is restrained when the quantity of salt present exceeds  $\frac{1}{2}\%$  and, in any case, the coloration of its colonies is never sufficiently distinctive.

(R) *Boric Acid* added to agar in quantities up to 2% allows the growth of *B. diphtheriae*, *Staphylococci*, *B. Hofmanni* and certain *Streptococci* to take place but it inhibits markedly growth of *B. subtilis* and *B. megatherium*. Thus with a mixture of *Streptococci*, *Staphylococci* and *B. subtilis* (subcultured from an abscess on plain nutrient agar), the proportion of *B. subtilis* to other organisms was over 1000 to 1 and it was with difficulty that the *Streptococci* and *Staphylococci* were recognised. Subcultivation at 37°C. on agar containing 2% boric acid

showed an approximately equal distribution of *B. subtilis* and the various forms of cocci. On making further subcultures, on agar containing 4% boric acid, the difficulty was reversed, *B. subtilis* becoming hard to find, the culture appearing to be a mixture of *Staphylococci* and *Streptococci* only.

*Effect of Combined Salts.* As a result of the foregoing experiments bile salt 1% (=B), potassium sulphocyanide  $\frac{1}{2}\%$  (=A), potassium cyanide  $\frac{1}{4}\%$  (=E), and potassium ferrocyanide  $\frac{1}{4}\%$  (=O), and glucose  $\frac{1}{2}\%$  (=g) were added in various combinations to neutral-red agar. Where KCN (=E) was added special care was taken to neutralise the alkalinity by the addition of HCl. The medium was plated and surface streak cultures made and incubated at 37° C.

*Combinations tested:* BE, BEg; BA, BAg; BEO, BEOg; BO, BOg; BOA, BOAE.

*Organisms sown:* *B. diphtheriae*, *B. Hofmanni*, *B. megatherium*, *B. subtilis*, *Micrococcii*, *Torulae*, and *B. coli*.

*Results:* Bile salt (B) inhibited greatly the growth of *B. subtilis* and *B. megatherium*. Potassium cyanide (E), except when combined with glucose (g), inhibited for a time the free development of *Torula* which, on the medium containing the combination BEOg, gave deep red colonies.

Where *B. diphtheriae* was present the combinations BO, BAO; BEOg and BEO, BAOg, BOg gave red colonies when incubated at 37° C. for 24 hours.

The rapidity of acid production by the several media is in the order mentioned.

At 20° C. for 72 hours the coloration of colonies changed to brownish-yellow. With BO and BAO combinations acid production by the colonies of *B. diphtheriae* occurred in 12-18 hours, much earlier than with any of the remaining organisms tested, and, as a result of making surface streak inoculations of various artificial test mixtures of organisms on media containing BO or BAO combinations of salts and incubating at 37° C., it appeared that wherever reddish colonies developed in 12 hours *B. diphtheriae* was invariably found to be present. The absence of red colonies, however, was not evidence of the absence of *B. diphtheriae*, and it became necessary to continue the experiments.

(I) Thereupon *Potassium Ferricyanide*  $\frac{1}{4}\%$  (=l) and lactose 1% (=l) were added as variants to the combinations of neutral-red agar already cited. Bile salt (=B) was constantly present in each combination.

*Combinations tested:* BO, BI; BOg, BIg; BOE, BIE; BAO, BAI; BAOg, BAIG; BAOE, BAIE; BOgl, BIgl; BAOgl, BAIgl; BEO, BEI; BEOg, BEIg; BEOl, BEII.

Organisms tested (surface streak cultures incubated at 37° C.): *B. coli*; mixture of *Torulae* and *B. Hofmanni*; mixture of *Cocci* and *B. Hofmanni*, and *B. diphtheriae*.

The following points were noted :

(1) The strain of *B. diphtheriae* employed did not grow on any of these combinations by the 88th hour.

(2) A large number of involution forms of Hofmann's bacillus were observed in subcultures during the early period of incubation.

(3) Whenever a medium contained both the salts AI *Torulae* alone flourished on incubation, all the remaining organisms tested—*B. coli*, *B. Hofmanni* and *Cocci*—being rigidly inhibited.

(4) Later, at 20° C., the growth of *Torulae* on media containing any of the following combinations: BIg, BAIG, BIgl, gave rise to colonies having a deep prussian-blue coloration in centre with reddish periphery. On examination with the lower power ( $\times 60$ ) crystals resembling calcium oxalate were observed associated with a dark amorphous deposit in the immediate neighbourhood of the growth.

(5) Rapidity of production of alkalinity by colonies of the various organisms tested depended on the combination of salts added to the media. The following was the order of appearance of alkalinity in the combinations tested: (a) BEI. (b) BEO. (c) BAO or BAI and lastly (d) BO or BI.

The foregoing combinations were repeated, save that the percentage of salts added was reduced by one half in each case, with the result that in those combinations from which bile salt had been excluded *B. diphtheriae*, when present, again flourished, giving rise to colonies of varying tints, the combinations I, IA, and IO yielding distinctive pinkish colonies. The proportion of I (i.e. potassium ferricyanide) added varied from  $\frac{1}{8}\%$  to  $\frac{1}{16}\%$ .

These results have not been regarded as sufficiently satisfactory to warrant the use of media containing any of the foregoing combinations of salts for the routine diagnosis of *B. diphtheriae*, and in place of agar sheep's blood-serum has been used.

## II. COAGULATED SERUM MEDIA.

Recently clotted sheep's blood was deprived of the exuded serum which then was placed in a refrigerator and allowed to sediment for three days. The clear supernatant straw-coloured fluid was at length carefully drawn off clear of the deposit of red blood corpuscles, for it is essential that the serum be free of such corpuscles before use.

To this serum there is added 25 % nutrient broth, 1 % glucose, and 1 % of 1 % watery neutral-red as indicator.

(Where a refrigerator is not available 2 % chloroform may be added to the serum which is then placed in a tall glass cylinder and sedimentation allowed to proceed at air temperature. The subsequent process of sterilisation serves to expel the added chloroform.)

To this serum were added various concentrations of salts as with agar, and the reaction adjusted to faint alkalinity so that on coagulation the medium has a yellowish-buff appearance.

*Bile Salt; Potassium Cyanide.* It quickly became apparent that the addition of either of these two salts to serum formed a useless mixture, for a substance was produced which either did not coagulate on heating, or, if coagulated, became soft and jelly like and finally liquefied in the process of sterilisation. These two salts were therefore discarded.

*Test Organisms:* *B. diphtheriae*, *B. Hofmanni*, *B. megatherium*, *B. subtilis*, *Staphylococcus pyogenes aureus*, other *Staphylococci*, *Streptococci*, and *Torulae*, all recently isolated from cultures derived from throat swabs.

Nomenclature as with agar media generally, e.g.

'A' = 1 % KCNS added to medium,

'R' = 1 %, R 2 = 2 %, R  $\frac{1}{4}$  =  $\frac{1}{4}$  %, R  $\frac{1}{8}$  =  $\frac{1}{8}$  % respectively, boric acid added.

'I' = 1 %, I  $\frac{1}{2}$  =  $\frac{1}{2}$  % respectively of potassium ferricyanide added.

On media containing combinations AR  $\frac{1}{8}$  and AR  $\frac{1}{2}$ , colonies of *B. diphtheriae* gave distinctive pinkish growth as compared with a yellowish or yellow-brown growth given by the remaining test organisms. Similarly with medium containing I  $\frac{1}{2}$ , colonies of *B. diphtheriae* showed a distinctive pink coloration. Coagulated serum to which potassium ferricyanide has been added is, however, somewhat soft and friable and easily torn with slight pressure.

At this stage it became apparent that mere acid production with specific coloration, pink in the case of neutral-red media, is not the sole desideratum to be fulfilled by a method to be used for the ready recognition of the presence of the diphtheria bacillus either in pure culture or admixed with other organisms. Rather a large number of varieties or strains of *Staphylococci* derived from throat cultures give rise, on incubation, to acid in the presence of glucose, and the problem becomes narrowed down to the evolution of a medium which, while specifically favourable to the growth of *B. diphtheriae*, will on incubation produce specific changes other than colour-change due to mere alteration in reaction.

A medium obtained by adding to neutral-red-broth-glucose-serum 1% of potassium sulphocyanide and thereafter coagulated presents, with regard to the following organisms, certain phenomena which become noticeable on incubation of surface streak cultures at blood-heat. (This particular combination is hereafter referred to as the 'A' medium.)

(a) *B. diphtheriae*: Acid production in the first 18 hours. Growth not raised, colonies discrete, very small, barely distinguishable. The surface of the growth more nearly resembles ground glass in dryness and general appearance. The colonies are pink. The medium becomes pink and a bluish-pink tint diffuses out into the medium far beyond the immediate limits of the colonies. These phenomena are particularly well marked with subcultures taken direct from the throat or nose.

The subsequent behaviour of the culture at 20° C. is of interest in that the pink tint continues to diffuse throughout the medium and there appears to be a slight diminution of intensity of colouring in the immediate neighbourhood of the growth.

With subcultures taken from laboratory strains which have for many generations been propagated solely in artificial culture the foregoing phenomena—reddish coloration of colonies followed by specific coloration of medium with diffusion of tint throughout the medium—are not so intense.

(b) *Staphylococcus pyogenes aureus*: In 18 hours localised growth; colonies raised, tinted, distinguishable, discrete; acid production variable and depending entirely upon the strain employed. If there be marked acid production by the colonies, a pink colour develops in the medium. This coloration is usually confined to the immediate neighbourhood of the colonies. With some varieties of *Staphylococci*, however, diffusion takes place into the surrounding medium, and, in such cases, the diffusing

tint is usually patchy or streaky in distribution. The bluish tint so characteristic of *B. diphtheriae* is absent, nor with colonies of *Staphylococci* does diffusion of tint occur so readily into the surrounding medium at 20° C.

(c) *Torulae* in 18 hours: Acid production variable, growth raised, usually creamy. Medium may be tinted pink and if diffusion occurs it is but slight and of a faint pink tint.

(d) *B. megatherium* in 18 hours: Creamy growth, browning of medium in neighbourhood of colonies.

(e) *B. subtilis* in pure culture in 18 hours: Easily recognisable crinkly growth—membranous. Acid production, if marked, gives rise to a brownish-pink coloration and if diffusion occurs the coloration is pink. There is no trace of blueness. With continued incubation there may be liquefaction of the medium.

During the first 18 hours' incubation of a mixture of organisms containing either *Staphylococci* or *B. diphtheriae* in addition to *B. subtilis* there is some inhibition of growth of *B. subtilis* and apparently little restraint of growth of the remaining organisms. If incubation be continued for another 24 hours however, there is a marked acceleration of growth of *B. subtilis* giving rise to the usual membranous appearance as seen with the pure culture of that organism, and if incubation be continued liquefaction of the medium usually occurs.

(f) *B. Hofmanni* in 18 hours: Yellowish growth well marked. The medium becomes markedly yellow and on continued incubation there is a general yellowish diffusion of tint (=alkalinity) throughout the medium.

(g) *Streptococci* derived from throat cultures: Very slight acid production, if any, and the pink tint rarely diffuses away from the colonies. When diffusion occurs the tint spreads in a patchy or streaky manner in the immediate neighbourhood of the several colonies. This medium seems to favour the free growth of *Streptococci* very markedly.

The 'A' medium has been used as a routine measure for the purpose of detection of the diphtheria bacillus in a very large number of throat cases, and, as a result of experience, it seems possible to say that where no bluish-red coloration appears after 18 hours' incubation of the inoculated medium *B. diphtheriae* is absent. Conversely it is possible, after a very short experience, to differentiate by means of the several tints produced the various organisms described, always taking into consideration the naked-eye appearances of the culture after the first 18 hours of incubation at blood heat. *B. diphtheriae* seems to be specially

favoured by this medium, and next in order *B. Hofmanni* and *Streptococci* when present in pure culture.

Even in the case of admixtures of organisms there is shown on incubation a decided preferential action favouring first of all the diphtheria bacillus, and next in order *B. Hofmanni*, the *Staphylococci* and *Streptococci*. In pure culture *B. subtilis*, *B. megatherium* and *Torulae* flourish apparently unrestrained, but if they be present as a slight or accidental contamination of the pathogenic organisms cited—the differentiating and selective action of the medium in favour of such pathogenic varieties, continuing markedly during the first 18 hours of incubation, is sufficient to enable a ready naked-eye diagnosis to be made at the end of that period. On continued incubation the non-pathogenic varieties tend to overgrow and mask by their effects the presence of the pathogenic forms.

Where difficulty is experienced in distinguishing the several tints at close range it is often of considerable service to view the cultures at some little distance when the bluish-pink variety of tint peculiar to colonies of *B. diphtheriae* becomes specially noticeable.

*Calcium Chloride* added to the medium ('A') has the effect of increasing the intensity of the tints produced, and, in addition, the characteristics of the several growths cited appear to be more marked. The presence 1% of the salt seems sufficient for the purpose. If, however, 2% of the salt be added there is a marked restraint of growth of *B. Hofmanni*. In the case of the various *Staphylococci* production of the natural pigment in the raised colonies seems to be unusually well marked. (This latter phenomenon may have some relationship to the fact that the local water used for general laboratory work is excessively soft and deficient in lime salts, and pigment production by the various *Staphylococci* on ordinary agar media is usually not well marked.) If  $\text{CaCl}_2$  be present in quantities exceeding  $2\frac{1}{2}\%$  there is marked restraint of growth of *Torulae* and *B. diphtheriae*.

Of the groups of four organisms cited, *Staphylococci* are least restrained by the presence of  $\text{CaCl}_2$ , but are altogether inhibited when the quantity present exceeds 3%.

Calcium chloride causes the medium to assume a firmer and whiter appearance on coagulation and the medium seems to 'keep' better for longer periods. The apparent intensification of the several tints and the better marked characteristics generally of the various growths may be apparent rather than real and due to the increased whiteness of the medium.

Glycerine added to the extent of 5% to the 'A' medium appears to have the effect of delaying drying of the medium when prepared as slopes in tubes or in plates, and, moreover, adds to the nutritive and selective value of the medium so far as the diphtheria bacillus is concerned. Occasionally the intensity and rate of diffusion of the specific colours produced by the colonies of the various organisms described are better marked on glycerine 'A' medium than on the plain 'A' medium. Neither with the glycerine 'A' medium nor with the calcium-chloride 'A' medium have there been sufficiently extensive tests carried out with organisms derived directly from throat swabs to indicate with any degree of certainty that either medium is of greater utility than the simple 'A' variety. There is an indication for the use of calcium-chloride medium on occasions when it is necessary to exclude *B. Hofmanni* on incubation from a mixture of organisms in order to facilitate the diagnosis of other organisms present and more particularly to detect the presence of *B. diphtheriae*.

Latterly to the 'A' medium there has been added but  $\frac{1}{2}\%$  of glucose, and on the whole this modification has proved of greater service in that the tint produced by colonies of *B. diphtheriae* is more readily distinguished from those produced by colonies of other organisms especially during the first 18 hours of incubation.

Throughout these experiments the presence of *B. diphtheriae* has been confirmed by microscopic examination. A wet method has been used as follows: Films are made on slides, fixed, dried by heat, and cooled and covered with a drop of  $\frac{1}{2}\%$  watery-methylene-blue for a few seconds. A cover-slip is placed over the drop and blotting paper pressed heavily on. The organisms are then examined by means of an oil-immersion lens and their characteristic morphological appearances and arrangement noted. Thereupon while still under observation a drop of  $\frac{1}{2}\%$  acetic acid is placed at the side of the cover-slip and differentiation shortly occurs, the beaded appearance of the organisms giving way to decolorisation with the appearance of dark granules—polar bodies—characteristic of the bacilli. I have occasionally been misled by the appearance of organisms resembling morphologically *B. diphtheriae* and indistinguishable therefrom, such organisms being a contamination of the watery-methylene-blue first added. Such organisms however are not fixed on the slide and on careful focussing they may be seen to be floating immediately below the lower surface of the cover-slip. In such cases it is always better to renew the watery-methylene-blue in use at short intervals and sterilise the bottle on each occasion.

The polar bodies of *B. diphtheriae* grown on glycerine 'A' media seem specially well marked, and the bacilli are more regular in shape, there being less tendency to the production of involution forms.

Certain strains of acid-producing *Staphylococci*, especially those obtained after repeated subculture on the ordinary laboratory media,

				Incubation at 37° C. for 18 hours		At 37° C. for 36 hours	
		Control, uninoculated colour		<i>B. diphtheriae</i> (No. 1633)	Marked and producing <i>Staphy-</i> <i>lococcus</i> (No. 1900)	<i>B. diphtheriae</i> (No. 1633)	<i>Staphylococcus</i> (No. 1900)
Medium, Blood serum + 25 % Broth + Glucose $\frac{1}{2} \%$ + Neutral red	$\frac{1}{2} \%$ Boric acid = R $\frac{1}{2}$	Bright yellow		Pink growth ; some little dif- fusion of tint into surround- ing medium	As with <i>B. diph.</i>	Growth colour as at 18 hrs., but greater diffu- sion	As with <i>B. diph.</i>
1 % Boric acid = R 1	Faint pinkish yellow			Pink growth ; some diffusion	Slight localised pink growth ; no diffusion of tint	As at 18 hrs., but increased diffusion	As at 18 hrs., slight diffusion
2 % Boric acid = R 2	Faint pink tint			Pink growth ; very slight dif- fusion	Localised pink growth ; no diffusion	Pink growth ; some diffusion	Pink growth ; very slight dif- fusion
1 % KCNS = A	Yellow	...		Pink growth ; some bluish- pink diffusion	Pink growth ; very little dif- fusion	Overgrown with <i>B. subtilis</i>	Pink growth ; more diffusion
AR $\frac{1}{2}$	...	Yellow	...	Pink growth + diffusion	Pink growth ; slight diffusion	Pink growth + bluish - pink diffusion	Pink growth + pink diffusion
AR 1	...	Faint pinkish- yellow tint		Pink growth ; slight diffusion	As with <i>B. diph.</i>	Increased growth and diffusion	As with <i>B. diph.</i>
AR 2	...	Faint yellowish pink		Faint pinkish growth ; free broth in tube acid	Yellow - pink growth ; free broth alkaline	Increased pink tint	As at 18 hrs.
Potassium fer- ricyanide $\frac{1}{2} \%$ = I $\frac{1}{2}$	Yellow	...		Deep pink growth ; gen- eral diffusion of tint	Yellowish pink growth ; slight diffusion of tint	As before	... As before, but increasing dif- fusion of tint
K <sub>3</sub> FeC <sub>6</sub> 1 % = I 1	Faint red pink (cerise)			Pink growth + diffusion of tint	Whitish growth on pink base + some diffusion	As before	... Tint and growth yellowish
K <sub>3</sub> FeC <sub>6</sub> 2 % = I 2	Brown pink (medium soft)			Pink growth ...	Brownish pink growth	Colour ap- proaching coc- cus growth	As before
AI $\frac{1}{2}$	...	Yellow	...	Deep pink growth and diffusion of tint	Yellow - pink growth + dif- fusion	As before	... Yellowish tint disappearing but difference still marked
AI 1	...	Brownish - yel- low (medium soft)		Rich pink growth and bluish - pink diffusion	Yellow - pink growth on a dull cerise base	As before	... As before, but growth becom- ing pinker ; difference be- tween cultures still very mark- ed.
AI 2	...	Greenish - yel- low brown		Reddish growth + colour of me- dium	As colour of me- dium	Reddish fading	tint As before, very little differ- ence between cultures
Potassium fer- rocyanide 1 % = O	Bright yellow			Very faint pink growth ; very slight diffusion of colour	Pink growth ; slight pink dif- fusion	As before	As before
AO	...	Yellow	...	Pink growth and some pink diffusion	Slight pink growth & slight localised dif- fusion	Increased growth and diffusion	Increasing depth of tint and diffusion

The value of the media in differentiating growths by means of colour reaction (including diffusion of tint through media) is in the following order:—A, AI  $\frac{1}{2}$ , I  $\frac{1}{2}$ , AI; RI, AR  $\frac{1}{2}$ , AO; I 1, R 2; the first three being specially valuable during the first 18 hrs. of growth, and the two last showing up differences after 36 hrs. incubation.

occasion some difficulty in naked-eye diagnosis when incubated on the modified 'A' medium, and the tints produced approximate very closely to those furnished by the colonies of *B. diphtheriae* at the 18th hour of incubation. Further incubation up to the 24th hour is, as a rule, sufficient to overcome this difficulty.

The following experiments show in greater detail some methods which serve to differentiate the appearances of colonies of acid-producing *Staphylococci* from those of *B. diphtheriae* on 'A' and other media.

It is somewhat remarkable that potassium ferricyanide when present to the extent of 1% in blood serum does not inhibit the growth of *B. diphtheriae* or of certain *Staphylococci*, whereas when the quantity exceeds  $\frac{1}{8}\%$  in nutrient agar complete restraint of growth occurs.

To complete the series Boric acid (= R) from  $\frac{1}{2}\%$  to 2% has been added to neutralised-glucose-bouillon-serum both with and without sulphocyanide of potassium and various organisms sown with the following results. (Period of incubation, three days at 37° C.)

*B. Hofmanni*: on media R  $\frac{1}{2}$ , and AR  $\frac{1}{2}$ , fair growth, many discrete colonies; on media R 1 and AR 1 slight growth only; on media R 2 and AR 2, very slight growth indeed.

*Staphylococcus p. aureus*: pink growth with some pink diffusion on all the combinations tested.

*B. megatherium*: some growth on all the combinations tested; many spores present; some pink diffusion.

*Torulae*: very slight growth indeed on media containing R  $\frac{1}{2}$  or R 1.

I wish to express my deep indebtedness to Dr T. Thomas Rankin, Senior Medical Officer of the Leeds City Fever Hospitals, for the trouble and care he has taken in applying independently some of the foregoing media to the diagnosis of *B. diphtheriae* and associated organisms in cases under his care.

Up to date over 6000 tests have been made, 3000 of which have been with the modified 'A' medium alone. With this modification there has not been a single instance in which it was not possible to diagnose the presence or absence of the diphtheria bacillus without the aid of a microscope, and solely by means of the naked eye. In particular, it is possible to state that when the specific coloration is absent after 18 hours' incubation at blood heat, the absence of *B. diphtheriae* may safely be assumed.

To dispense with the microscope in the routine examination of cases of suspected diphtheria—either for the detection of carrier cases or in the examination of swabs from likely cases of diphtheria among the general population—would mean a saving of at least 90 %; while in the routine examination of cases of diphtheria already isolated, either for the purpose of confirmation of diagnosis or with a view to the discharge of patients apparently convalescent of the disease, there would be a saving at least of 50 % of the time and labour now involved.

For the latter purpose as well as for the routine detection of cases of post-scarlatinal diphtheria in scarlatinal isolation wards the modified 'A' medium seems specially suitable.

## UPON THE INOCULATION OF MATERIA MORBI THROUGH THE HUMAN SKIN BY FLEA-BITES.

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*Introductory.* It has long been supposed that fleas may play a part in the transmission of disease, but no evidence which would clearly incriminate fleas has been forthcoming until the last few years. It has been clearly established within the last fifteen years that other blood-sucking parasites may convey disease, that mosquitoes propagate malaria, yellow fever, filariasis; that certain brachicerous flies propagate trypanosome diseases; that ticks transmit Rocky Mountain fever, African relapsing fever, piroplasmosis in cattle and other animals; and that bed-bugs convey relapsing fever.

Fleas, like the other blood-sucking parasites above enumerated, frequently leave and change their hosts, and not uncommonly pass from animals to man, and *vice versa*. Hence it appears desirable to enquire into the possibilities of fleas as disease-transmitters.

Papers on the transmission of *Trypanosoma lewisi* by rat-fleas have been recently published by Nuttall (1908), by Minchin and Thomson (1910), and by Strickland and Swellengrebel (1908-1910).

The conclusion that fleas were an essential factor in the spreading of plague was drawn by Simond in 1898; the same conclusion was reached by Hankin (1898), and by Liston, Ashburton Thompson (1902), from a careful examination of the epidemiological evidence afforded by outbreaks at Sidney, also concluded that plague was transmitted by fleas. Verbitski (1904) in Russia performed numerous experiments demonstrating the ability of fleas to transmit plague among rats. Since 1906 the Advisory Committee for Plague Investigation in India appointed by the Royal Society, the Lister Institute, and the Secretary

of State for India, has published a mass of observational and experimental evidence to the effect that plague is conveyed both to rats and to man almost exclusively by flea-bites, other—possible—modes of infection being very rare. That fleas may transmit plague to other animals has also been shown by the Advisory Committee (1906-1908), and by McCoy (1910) in America<sup>1</sup>.

Direct experiments with fleas on man have not been recorded.

Some enquiries related to the possibility of disease transmission by fleas were begun by the writer; the following experiments to determine the ability of fleas to inoculate *materia morbi* lying upon the skin form a fairly complete series in themselves.

*Method of experiment.* The fleas were kept in small glass tubes; they were fed on an average once a day; in this way they can be kept alive for several weeks. The experimental bites were mostly made on the flexor aspect of the arm but some were made on the front of the thigh and some on the calf. The inoculable material was spread on a small area of skin until the latter was moist; one of the flea-tubes, with open end, was then inverted over the spot. The fleas bit through the film of material. Occasional clean bites were made for comparison. In other experiments the fleas were allowed to bite clean skin, and the disease material was immediately afterwards rubbed over the site. At first each presumed bite was confirmed by examination of the site with a low power of the microscope, but the writer shortly became able to recognise a bite with certainty by the attitude and behaviour of the flea, apart from the appearance of the skin. The fleas generally bit within a minute or two of being applied to the skin, so that the film was still moist; in a few instances of unusual delay the film had dried. Control experiments through wet and dried films however gave uniformly positive results.

*The materia morbi.* Three kinds of material were used, (a) Tuberculin, (b) Vaccine Lymph, and (c) an Emulsion of a *Staphylococcus albus*.

(a) The *Tuberculin* used was that prepared by Allen and Hanbury for von Pirquet's diagnostic cutaneous reaction. It consists essentially of the concentrated filtrate from a broth culture of human tubercle bacilli. In from twelve to twenty-four hours after it has been inoculated

<sup>1</sup> If we add that Cysticerci of Cat, Dog and Rat Tapeworms have been found in fleas, we have summarized about all that is at present known of fleas in relation to disease. Sir Jonathan Hutchinson (1909) suspects that fleas may occasionally convey Syphilis, but the evidence is exceedingly slight.

into the skin of a susceptible person a papule measuring 2-3 mm. in diameter appears at the site of inoculation. It is vivid red in colour, and is surrounded by a bright pink areola about 1 mm. wide. The patient is also conscious of some soreness. A few hours later one or more vesicles may appear. The reaction is generally at its height about the end of the second or third day, after which it slowly subsides, being usually recognisable up to three weeks later; it often gives rise to a slight pigmentation which fades even more slowly; simple needle-pricks of a similar depth, made as controls, disappear completely in about two days.

(b) *Vaccinia* was selected as being an actual disease of kindred nature with the acute specific fevers, which is very highly inoculable and which may be imparted with safety. Its behaviour is exceedingly regular; and positive results, which are quite characteristic, follow with great certainty except in highly immune persons. It is therefore eminently suitable for experiments of this kind; its chief drawback is that a positive result renders the subject useless for further experiment for a considerable time. Ordinary government glycerinated lymph was used.

(c) From a small receding pustule on the neck of the writer was cultivated a Gram-positive *Staphylococcus*, which grew on agar in dirty-white, moist, fairly robust colonies of a rather gluey consistence. From about 36-hour agar growths emulsions were made in normal saline, of 2,000,000 per cubic millimetre. When these emulsions were pricked into the skin, a deep red papule formed in about 24 hours; it was surrounded by a zone of inflammation about a centimetre across. In about 36 hours the centre became pustular. At the end of each series of experiments the emulsions were recultured and gave robust growths in each case. These experiments were only performed on the writer.

*The fleas.* The fleas were all obtained in a general hospital. About 80 were used in all; of which about 35 were male, and about 45 female. Of twenty-eight which were examined with a view to identification 27 conformed to the description of Jordan and Rothschild (1908) for *Pulex irritans*, and one to that of *Ceratophyllus fasciatus*.

*The persons.* There are great differences between individuals in the local reaction with which they respond to flea-bites. Of the three persons of the experiments, L. K., H. W., and C. W. (the writer), the two latter exhibit the minimum of reaction, consisting of a pale pink areola about three millimetres in diameter, which fades in a few hours, and is unaccompanied by sensation. L. K. is at the other end of the

scale, and a bite is followed after a few minutes by a bright red flush several centimetres across, in the centre of which a raised white wheal about a centimetre wide appears after a few minutes; there is a sensation of extreme irritation. The flush is a day or two in fading.

*The controls.* Control experiments were repeatedly made, with ordinary precautions against sepsis, by laying a film of the material on the skin as usual and then pricking through it with the finest sewing needle made, the diameter of the shaft of which was 0·37 mm.

The proboscis of *Pulex irritans* measures 0·025 to 0·03 mm. at its greatest diameter, and 0·015 mm. at 0·02 mm. from the point. The needle, though a very fine one, is thus, speaking quite roughly, more than twelve times the size of the flea's proboscis.

The pricks were made with a single sharp movement; their depth was exceedingly slight, so that on many occasions the writer doubted whether the needle-point had entered the epidermis at all. Nevertheless of 48 such pricks only one failed to give a decided positive reaction.

#### THE EXPERIMENTS.

##### A. With Tuberculin.

(a) The fleas biting through films of Tuberculin.

1. C. W. 80 bites were made. 75 gave completely negative results, one was doubtful, and four were followed by positive reactions.

The four positive results all occurred together, and were from a series of five bites made just above the patella.

60 bites were on the arm and wrist, 29 were on the leg. 39 were by male fleas, and 41 by females.

Controls: 19 needle-pricks through Tuberculin films, 18 were positive, one doubtful (probably the needle did not penetrate).

Two pricks were made through films which had been dry for six hours, both were positive.

2. H. W. 12 bites. 10 negative, 2 positive.

Controls: 5, all positive.

3. L. K. 2 bites, both negative.

Controls: 3, all positive.

##### Summary.

94 bites were made, 87 gave no inoculation, one was doubtful, and 6 were followed by inoculation.

68 bites were on the arm and wrist, 26 on the leg. 45 were bites by male fleas, 49 by female.

Of the 6 inoculating bites, 4 were by males and 2 by females.

26 controls were made, 25 were positive, one was doubtful.

(b) Tuberculin rubbed over recent flea-bites.

1. C. W. 25 experiments, all negative.  
Controls, Tuberculin rubbed over needle-pricks.  
3 experiments, all positive.
2. H. W. 3 experiments, all negative.

B. *With Vaccinia.*

(a) The fleas biting through films of Vaccine Lymph.

1. C. W. 13 bites, all negative.

No controls were made, as the subject is required for further experiment. C. W. has been vaccinated once in infancy (two "very poor" marks), and is therefore presumably not immune to vaccinia.

2. H. W. 13 bites, all negative.

Controls, 3 needle-pricks were made through a vaccine film, each gave a small but characteristic vaccine pustule. H. W. has been vaccinated twice, once in infancy and once about seventeen years ago.

3. L. K. 11 bites, all negative.

Controls, 2 needle-pricks were made through a vaccine film, in five days small bright papules formed with slight areolae. The papules were visible three weeks later, but no vesication had occurred. Clean needle-pricks left no mark after two days. Two further pricks were made through vaccine films, no reaction followed. The controls are therefore taken as positive. L. K. has been vaccinated three times, the last about seven years ago.

Summary. 45 bites, none inoculated.

25 bites were on the arm, 20 on the leg. 19 were by male fleas, 26 by female.

5 controls, all positive. The fact that positive controls were obtained confirms the observation that the flea-bites had failed to inoculate.

(b) Vaccine Lymph rubbed over recent flea bites.

1. C. W. 4 experiments, all negative.
2. H. W. 5 experiments, all negative.

C. *With Emulsions of Staphylococcus albus.*

(a) The fleas biting through films of the emulsion.

C. W. 56 bites, all negative.

Controls, 15, all positive. Of these four were made through dried films.

15 bites were on the arm, 5 on the leg. 23 were by male, 33 by female fleas.

(b) The emulsion rubbed over recent bites.

C. W. 9 experiments, all negative.

Controls, 2, both positive.

*General Summary.* Fleas biting through films of inoculable materials, 196 experiments, 188 negative, 1 doubtful, 6 positive. No positive results with Vaccine Lymph and *Staphylococcus* Emulsion. Controls, 47. 45 positive, 1 doubtful.

Inoculable Materials rubbed over recent flea-bites, 46 experiments, all negative. Controls, 8, all positive.

*Discussion of result.* The circumstances of the experiments appear to be the most favourable for "mechanical" flea-bite inoculation, and yet inoculation only followed in about 3 per cent. of cases, and only with Tuberculin. In these few cases also it is not improbable that the Tuberculin may have penetrated some small patch of unusually deep desquamation, or some small abrasion, as invisible as many of the control needle-pricks were. It was not possible to say whether the reaction started from the precise point of penetration by the flea or not. Tuberculin when mixed with ointment is inoculable through the unbroken skin, as in Moro's diagnostic method.

The writer regards these six positive results as not necessarily due to the flea-bites. Without, however, pressing this opinion, it is obvious from the experiments that, with highly inoculable materials, and under apparently favourable circumstances, *Pulex irritans* only rarely effects inoculation when biting.

It may be suggested that as the amount of material, if any, introduced by a flea's proboscis must be exceedingly minute it may be too small to produce any recognisable effect. In order to test the possibilities of minute doses, experiments were made on C. W. with Tuberculin. Two methods suggested themselves, (1) to dilute the Tuberculin, using the same pricker, (2) to use a smaller pricker. There are difficulties in the way of the latter method in that if a pricker much smaller than the smallest commercial needle is used it is liable to break

#### EXPERIMENTS WITH MINUTE TUBERCULIN DOSES.

(1) Inoculations with diluted Tuberculin, needle as before.

Dilutions up to 1 in 10.

Reactions differing little if any from those with full strength.

Dilutions from 1 in 10 to 1 in 50.

Reaction slightly less than with full strength.

Dilutions from 1 in 50 to 1 in 100.

Reactions characteristic but smaller and diminishing with the dilution.

Dilutions from 1 in 100 to 1 in 150.

Pricks still easily recognisable from clean controls, especially after the lapse of a day or two.

- (2) Full strength Tuberculin pricked through by nettle-hairs.

The nettle hairs are fairly regular cones 1 to 1·3 mm. long ; their diameter is 0·085 mm. at the base, 0·06 mm. in the middle, and 0·014 mm. just below the terminal knob, which is broken off as the hair enters the skin ; at 0·02 mm. behind the knob the diameter is 0·018 mm. Thus, to make a rough comparison between the proboscis of *Pulex irritans* and the nettle hair, the latter is about three times as long, it is about the same diameter near the point, but farther back it becomes 2-3 times as thick.

The skin-reactions from nettle-stings and those from Tuberculin can run their courses concurrently, without apparently influencing each other.

With "slight" nettle-stings, i.e. less than about ten hairs penetrating within two or three mm., no trace is left after the lapse of a day. With "severe" stings, more than about twenty hairs piercing in close proximity and producing confluent wheals, there are often several small pink papules on the following day which are not at once distinguishable from small Tuberculin reactions. Upon development however a distinction appears, as the nettle-sting papules fade completely in about twenty-four hours more, while the Tuberculin papules steadily increase in size and characteristics for several days.

The experiments showed that when single hairs, obtained by cutting out a piece of leaf bearing a solitary hair, were pricked through a Tuberculin film there was no wheal, but a Tuberculin reaction followed, which, though small, passed through the usual stages in the usual times.

When two or three or more hairs were passed through in close proximity so as to give a confluent wheal, a correspondingly greater reaction followed.

(Note. These two sets of experiments were carried out at an interval of some months after the others, but the appearance of the usual controls did not suggest that there had been any great change in the susceptibility of the subject to Tuberculin.)

upon, or within, the epidermis ; the latter event is liable to cause a spot of inflammation which obscures the issue, but in its absence there may be complete uncertainty as to whether the pricker has penetrated at all. An opportunity is offered however by the hairs of the stinging-nettle, which while very minute announce their penetration to sensation and by the shortly subsequent appearance of the well-known skin reaction.

These experiments show that a recognisable reaction is obtainable with an amount of Tuberculin which is exceedingly minute, and which is comparable to the amount which might be carried into the skin by a flea's proboscis. The conclusion is, therefore, that the failure to obtain inoculation with flea-bites is not attributable solely to the smallness of the piercing-organ.

*Bearing on plague.* The foregoing experiments derive interest from their bearing on the transmission of plague by fleas. It has been

shown by the Indian Commission (1907, p. 405) that fleas which have fed upon plague animals are liable to deposit faeces containing abundant virulent plague bacilli upon the skin of their host<sup>1</sup>. Simond (1898) had suggested that plague was transmitted by fleas subsequently biting through such faeces, or by the faeces being rubbed into the bite holes. The Indian Committee examined experimentally a number of presumably possible modes of transmission by fleas, and failed to find evidence in favour of any but the above two. (1907, pp. 415 *et seq.* The reader is also referred to this place for a discussion on the mechanism of transmission.) Successful inoculations were obtained with guinea-pigs by allowing fleas to bite them through films of plague culture, and also by smearing cultures over recent bites. Verjbitski (1908, p. 183) also performed the latter experiment, with variations, upon rats, obtaining positive results in about 50 per cent. of cases. Neither mention whether precautions were taken against the animals scratching themselves.

In applying the here recorded experiments to the question of plague transmission it is necessary to refer to differences in each of the factors of the two sets of experiments.

(a) While the writer, as shown on p. 292, used *Pulex irritans* almost entirely, the Indian Committee used for their similar experiments *Xenopsylla cheopis* (Rothschild)<sup>2</sup>. The differences between the two fleas in vital anatomy are so slight that they are not likely to affect the result. According to Jordan and Rothschild (1908, p. 17) the piercing organ of *X. cheopis* is somewhat longer and slenderer than that of *P. irritans*, but it is not obvious in which direction this might tend to act. Also, in the recorded work upon the general fact that fleas are concerned in plague transmission, no distinction between different species of fleas has been indicated, beyond their affinities for different hosts and the consequences deducible therefrom.

(b) Instead of *B. pestis* in various media, the writer used the above mentioned three materials. It still may be suggested that the flea's proboscis may carry in a quantity of material which is so slight as to be below the limit of effectiveness of the writer's materials, but

<sup>1</sup> The statement that defaecation frequently occurs during the act of biting has been questioned. The writer observed it 51 times in 241 bites of *Pulex irritans*. It appears to occur more frequently the shorter the intervals between the feeds; the intervals averaged about a day in this case. The proportion of defaecating females to males was about 2.5 to 1.

<sup>2</sup> Verjbitski worked with *Typhlopsylla musculi*, *Pulex irritans*, *P. canis*, *P. felis*. The flea used in the precise experiments here referred to does not appear to have been recorded.

which nevertheless may be sufficient to originate fatal plague. This however is very improbable, for, while the minuteness of effective Tuberculin doses has been indicated above, the most virulent cultures of *B. pestis* which Verjbitski was able to obtain required 0·001 cubic millimeters for a minimum lethal dose for rats. Now the volume of the whole proboscis of *Pulex irritans* is only about 0·00018 cu. mm., and the amount of material which it is likely to carry in with it when piercing the skin will be smaller still, i.e. very much smaller than Verjbitski's minimum lethal dose. The Indian Commission also (1907, pp. 346-8) showed with guinea-pigs that the smaller the dose of plague bacilli the less the effect produced. With very small doses no result was discovered.

(c) The plague experiments have been conducted on rats and guinea-pigs, the others on man. It is possible that differences exist between man and the experimental animals in this respect, but if this be so the conclusions from the experimental work on plague must be modified on being applied to man.

A further experimental difference exists in that with the writer the bites were nearly all single (six were double), whereas in the plague experiments they were mostly multiple. The Indian Commission (*loc. cit.*) succeeded only once in conveying infection to rats by a *single* flea-bite. Verjbitski failed to inoculate fatally with a single bite in four trials by smearing plague cultures over recent bites. He concluded that at least two bite-holes were necessary for a fatal inoculation in a rat, but he obtained fatal inoculations in guinea-pigs by smearing the cultures over one bug-bite (1908, pp. 183 and 197). The ability of one inoculation to influence another obviously depends on the overlapping of their spheres of influence. If the important action is confined to the immediate neighbourhood of the place of introduction, as is most probably the case with Tuberculin and Staphylococci, two inoculations will not affect each other, unless they occur very close together. Hence it is improbable that with the writer's materials the appearances, or lack of appearances, following a single bite will be altered by multiple bites, unless the latter are closely set. However, it is intended to carry out further experiments along these lines.

Although in no way affecting the conclusion that fleas are responsible for plague conveyance, the above recorded experiments are at variance with the view which at present meets with most acceptance regarding the mechanism by which fleas transmit the disease. It appears that yet further enquiry is needed.

*Acknowledgements.* The writer is under obligation to several correspondents for their ready courtesy. He also gladly acknowledges the assistance of Sister Lindsay, and of many members, too numerous for individual mention, of the Nursing Staff of Queen's Hospital, Birmingham.

He is specially indebted to his sister, Hilda Walker, and to Dr Lina Kurz for their willingness to assist in and be the subjects of a series of somewhat unpleasant experiments.

#### SUMMARY.

1. When fleas bit through films of Tuberculin, Vaccine Lymph, and Staphylococcus Emulsion lying upon the human skin, no inoculation was observed in 188 instances out of 195. In six of the experiments with Tuberculin, inoculation followed, which possibly was not due to the flea-bites. Controls gave positive results.
2. When the same materials were rubbed over recent flea-bites, no inoculations followed. Controls were positive.
3. A very minute dose of Tuberculin, such as might be conveyed by a flea's proboscis, will produce a recognisable reaction in a susceptible person.
4. The bearing of the results on the question of plague transmission is discussed.

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## FIFTEENTH INTERNATIONAL CONGRESS ON HYGIENE AND DEMOGRAPHY.

WASHINGTON, D.C., SEPTEMBER 23—28, 1912.

This Congress was originally announced to take place in 1910 (see *Journal of Hygiene*, 1909, vol. ix. pp. 347—356) but the date was subsequently altered to 1912. Certain changes in the organization have been made, as appears from a notice which has recently reached us. This notice states that:

The President of the United States will be the Honorary President of the Congress. Dr Henry P. Walcott, President of the State Board of Health of Massachusetts, will be the active President of the Congress.

The larger responsibilities in relation to public health and vital statistics in the United States are borne by the governments of States and cities. Therefore an Act of Congress has been passed, inviting the States, in terms as follows:

### [PUBLIC RESOLUTION—No. 12.]

[S. J. Res. 56.]

Joint Resolution Authorizing the President of the United States to invite the States to participate in the Fifteenth International Congress on Hygiene and Demography.

*Resolved by the Senate and House of Representatives of the United States of America in Congress assembled*, That the President of the United States be, and he is hereby, authorized and requested to extend an invitation to the governor of each State to appoint a state committee of not more than five members to cooperate with the committee on organization in planning and preparing the exhibition of the Fifteenth International Congress on Hygiene and Demography.

Approved, January 24, 1910.

In pursuance of this Joint Resolution, the Department of State, under date of February 4, 1911, has sent an invitation to the Governor of each State. From the Governor the invitation is intended to reach the mayors of cities which desire to participate in the Congress or the Exhibition. The letter of the Department of State said expressly that the invitation was meant to include the cities. State Committees will be organized in all the States.

The Department of State has created a Committee on Organization, composed of the fifty-three persons named below.

#### COMMITTEE ON ORGANIZATION.

- Chairman:* HON. HUNTINGTON WILSON, Assistant Secretary of State, Washington.
- ABBOTT, DR A. C., Professor Hygiene, University of Pennsylvania, Philadelphia.  
BELL, MR CHARLES J., Pres. Amer. Security and Trust Co., Washington.  
BEYER, DR HENRY G., Medical Director, United States Navy, Washington.  
BICKNELL, MR ERNEST P., Director National Red Cross Society, Washington.  
BIGGS, DR HERMANN M., Medical Director, Department of Health, New York City.  
BILLINGS, DR JOHN S., United States Army (retired), Librarian, Public Library, New York.  
BOARDMAN, MISS MABEL, National Red Cross Society, Washington.  
BROWN, DR ELMER E., Commissioner of Education, Washington.  
CHITTENDEN, PROF. RUSSELL H., Director Sheffield Scientific School, Yale University, New Haven.  
COMMONS, JOHN R., Professor Political Economy, University of Wisconsin, Madison.  
DEVINE, MR EDWARD T., Secretary Charity Organization Society, New York City.  
DIXON, DR SAMUEL G., Commissioner of Health of Pennsylvania, Harrisburg.  
DOCK, DR GEORGE, Professor Theory and Practice of Medicine, Washington University, St Louis.  
DURAND, HON. E. DANA, Director of the Census, Washington.  
FARNAM, HENRY W., Professor Economics, Yale University, New Haven.  
FARRAND, DR LIVINGSTON, Executive Secretary National Association Study and Prevention of Tuberculosis, New York.  
FAVILL, DR HENRY B., Professor Therapeutics, Rush Medical College, Chicago.  
FISHER, IRVING, Professor Political Economy, Yale University, New Haven.  
FULTON, DR JOHN S., Professor State Medicine, University of Maryland, Baltimore.  
GALLINGER, HON. JACOB H., United States Senator, Washington.  
GLENN, MR JOHN M., Director Russell Sage Foundation, New York City.  
GORIAS, DR W. C., Colonel, United States Army, Chief Sanitary Officer Isthmian Canal, Ancon.  
GULICK, DR LUTHER H., Director Department of Child Hygiene, Russell Sage Foundation, New York.  
HEO, DR ELMER E., Secretary State Board of Health of Washington, Seattle.

- HOFFMAN, MR FREDERICK L., Statistician, Prudential Insurance Company, Newark, N. J.
- JACOBI, DR ABRAHAM, Emeritus Professor Pediatrics, College Physicians and Surgeons, New York.
- KOBER, DR GEORGE M., Professor Hygiene, Georgetown University, Washington.
- KOREN, MR JOHN, Statistician, Boston.
- LATHROP, MISS JULIA, Hull House, Chicago.
- LOVETT, DR ROBERT W., Assistant Professor Orthopedic Surgery, Harvard University, Boston.
- McCAW, LIEUT.-COL. WALTER D., United States Army, Librarian, Surgeon-General's Office, Washington.
- MELVIN, DR A. D., Chief of the Bureau of Animal Industry, Washington.
- NORTH, MR S. N. D., Late Director of the Census, Washington.
- OLCOTT, HON. J. VAN VECHTEN, Ex-Member of Congress, Washington.
- OPHÜLS, DR WILLIAM, Professor Pathology, Leland Stanford University, San Francisco.
- PENROSE, DR CHARLES B., Advisory Board, Department of Health of Pennsylvania, Philadelphia.
- PROBST, DR C. O., Secretary State Board of Health of Ohio, Columbus.
- RAVENEL, DR MAZYCK P., Professor Pathology and Bacteriology, University of Wisconsin, Madison.
- SCHERESCHEWSKY, DR J. W., U.S. Public Health and M.H.S., Wash.
- SIMMONS, DR GEORGE H., Secretary of the American Medical Association, Chicago.
- SMITH, DR THEOBALD, Professor Comparative Pathology, Harvard University, Boston.
- STERNBERG, SURGEON-GEN. GEORGE M., United States Army (retired), Washington.
- VAUGHAN, DR VICTOR C., President of the State Board of Health of Michigan, Ann Arbor.
- WALCOTT, MR CHARLES D., Secretary of the Smithsonian Institution, Washington.
- WALD, MISS LILLIAN, Nurses' Settlement, Henry Street, New York.
- WELCH, MR ARCHIBALD A., Actuary, Phoenix Mutual Life Insurance Company, Hartford.
- WELCH, DR WILLIAM II., President State Board of Health of Maryland, Baltimore.
- WESBROOK, DR FRANK F., Professor Pathology and Bacteriology, University of Minnesota, Minneapolis.
- WILBUR, DR CRESSY L., Chief of Division of Vital Statistics, Census Bureau, Washington.
- WILLCOX, WALTER F., Professor Economics and Statistics, Cornell University, Ithaca.
- WILLIAMS, DR CHARLES F., Secretary State Board of Health of South Carolina, Columbia.
- WILLIAMS, DR EMMION G., Commissioner of Health of Virginia, Richmond.
- WYMAN, DR WALTER, Surgeon-General United States Public Health and Marine Hospital Service, Washington.

The Secretary-General is

DR JOHN S. FULTON, Army Medical Museum, Washington, D.C.

The Treasurer is

MR CHARLES J. BELL, President of the American Security and Trust Company, Washington, D.C.

The Committee on Organization has elected an Executive Committee, composed of the following members :

#### EXECUTIVE COMMITTEE.

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PROF. WALTER F. WILCOX, Vice-Chairman.

DR HENRY G. BEYER,

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GEN. GEORGE M. STERNBERG,

MR EDWARD T. DEVINE,

GEN. WALTER WYMAN.

The Congress is divided into nine sections as follows :

#### DIVISION I. HYGIENE.

##### SECTION I. HYGIENIC MICROBIOLOGY AND PARASITOLOGY.

*President* : PROF. THEOBALD SMITH, M.D., Harvard Medical School, Boston.

*Vice-Presidents* :

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##### SECTION II. DIETETIC HYGIENE; HYGIENIC PHYSIOLOGY.

*President* : DR RUSSELL H. CHITTENDEN, Sheffield Scientific School, Yale University, New Haven.

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*Secretary* : DR LAFAYETTE B. MENDEL, Sheffield Scientific School, Yale University, New Haven.

## SECTION III. HYGIENE OF INFANCY AND CHILDHOOD. SCHOOL HYGIENE.

*President* : DR A. JACOBI, Emeritus Professor Pediatrics, College of Physicians and Surgeons, New York.

*Secretary* : DR LUTHER H. GULICK, Director, Department Child Hygiene, Russell Sage Foundation, New York.

## SECTION IV. INDUSTRIAL AND OCCUPATIONAL HYGIENE.

*President* : DR GEORGE M. KOBER, Professor Hygiene, Georgetown University, Washington.

*Secretary* : DR ALICE HAMILTON, Hull House, Chicago.

## SECTION V. CONTROL OF INFECTIOUS DISEASES.

*President* : DR HERMANN M. BIGGS, Medical Director, Department Health, New York.

## SECTION VI. STATE AND MUNICIPAL HYGIENE.

(Including hygiene of houses, streets, water-supply and disposal of waste; legislative and administrative functions of boards of health.)

*President* : DR FRANK F. WESBROOK, Professor Pathology and Bacteriology University Minnesota, Minneapolis.

*Vice-Presidents* :

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DR CHARLES V. CHAPIN, Superintendent of Health, Providence.

*Secretaries* :

DR HERBERT D. PEASE, New York.

DR MARSHALL L. PRICE, Secretary State Board of Health of Maryland, Baltimore.

## SECTION VII. HYGIENE OF TRAFFIC AND TRANSPORTATION.

*President* : DR WALTER WYMAN, Surgeon-General, United States Public Health and Marine Hospital Service, Washington.

*Secretary* : DR JOHN W. KERR, Assistant Surgeon-General, United States Public Health and Marine Hospital Service, Washington.

## SECTION VIII. TROPICAL, MILITARY AND NAVAL HYGIENE.

*President* : DR HENRY G. BEYER, Medical Director, United States Navy, Washington.

*Vice-Presidents* :

PROF. F. G. NOVY, M.D., University Michigan, Ann Arbor.

LIEUT.-COL. HENRY P. BIRMINGHAM, United States Army, Washington.

*Secretary* : DR CHARLES N. FISKE, United States Navy, Washington.

## DIVISION II. DEMOGRAPHY.

## SECTION IX. DEMOGRAPHY.

*President* : PROF. WALTER F. WILLCOX, Professor Economics and Statistics,  
Cornell University, Ithaca.

*Vice-Presidents* :

DR JOHN S. BILLINGS, United States Army (Retired), New York.

MR FREDERICK L. HOFFMAN, Statistician, Newark, N.J.

*Secretary* : DR CRESSY L. WILBUR, Chief, Division Vital Statistics, Census  
Bureau, Washington.

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The Congress will be in session six days. There will be two general meetings, the official opening on Monday, September 23, and the closing session on Saturday, the 28th. The rest of the time will be taken up by the Section meetings.

The Exhibition is intended to illustrate the present status and recent progress of public hygiene and vital statistics, especially in the United States. Some foreign countries will send exhibits. The Exhibition will be kept open for a week or more after the Congress. The Director of the Exhibition is

DR J. W. SCHERESCHEWSKY, of the United States Public Health and Marine Hospital Service.

Active membership is open to persons interested in public health or vital statistics, on payment of the membership fee of \$5.00. Active members are entitled to take part in the discussions and to receive the published transactions.

Inquiries and applications for membership should be sent to  
The Secretary-General of the XVth International Congress on Hygiene and  
Demography, Army Medical Museum, Washington, D.C.

*Note.* The Regulations governing the International Congress of Hygiene and Demography, the lists of Members of the Permanent International Commission of the Congresses and of the British National Committee for the Congresses, are given in the *Journ. of Hygiene*, ix. pp. 349—356. Ed.

## PUBLICATIONS RECEIVED.

### BOOKS.

ADAMI, J. G. (1910). *The Principles of Pathology*. Volume I. General Pathology. Second Edition. Philadelphia and New York : Lea and Febiger. 1027 pp., 329 illustrations, 18 plates. 25 x 16 cm. Cloth.

ADAMI, J. G., and NICHOLLS, A. G. (1911). *The Principles of Pathology*. Volume II. Systematic Pathology. Second edition. Philadelphia and New York : Lea and Febiger. 1160 pp., 301 illustrations and 15 plates. 25 x 16 cm. Cloth.

It is difficult in the short space at our disposal to do justice to this monumental work, containing nearly 2,200 pages, 630 text-figures and 33 plates. *Volume I.*, of which Professor Adami is the sole author, is divided into two parts: Part I, Section I. Following upon an introduction, the chapters (I.—XV.) deal with the histology of the cell, its physiology, chemistry, growth, multiplication, adaptation, with cell and tissue differentiation, fertilization, biophoric hypothesis, inheritance. Section II. (14 chapters), *The Causes of Disease*, deals with—Inherited or acquired disease, marriage of consanguines, placental and foetal disease, monstrosities and abnormalities, post-natal acquirement of disease, exogenous intoxications, bodily states as causes of disease, predisposition and susceptibility. Section III., *The morbid and reactive processes* (10 chapters), deals with the local reaction to injury, the systemic reaction, immunization and immunity, systemic reaction through the nervous system. Part II. relates to tissue changes (27 chapters): hypertrophy, regeneration, grafting and transplantation, neoplasia (9 chapters), cysts, histolysis and cytolysis, degeneration and infiltration, calcification, calculi, pigmentation, necrosis, death, with an appendix on ultramicroscopic microbes. *Volume II.*, of which Professors Adami and Nicholls are joint authors, is divided into 10 sections and 23 chapters dealing with the cardiovascular, respiratory, alimentary and nervous systems, the ductless glands, the urinary, reproductive, tegumentary, muscular and osseous systems.

The work is one of first importance, and we congratulate the authors upon the completion of their arduous task. The form in which the volumes are issued leaves nothing to be desired.

AUSTEN, E. E. (1911). *A Handbook of the Tsetse-flies (Genus Glossina)*. 110 pp. 10 coloured plates (by A. J. E. Terzi), 24 text-figs. and 1 map. Printed by order of the Trustees of the British Museum. London: sold by Longmans & Co., 39 Paternoster Row, E. C.; B. Quaritch, 11 Grafton St, New Bond St, W.; Dulau & Co., Ltd, 37 Soho Sq., W. and British Museum (Nat. Hist.), Cromwell Rd, S. W.

All who are concerned with the study of blood-sucking flies, but especially those interested in Trypanosomiasis in Africa and its relation to tsetse flies, will welcome this valuable work, as it supersedes the author's *Monograph of the Tsetse-flies* (1903) which has been out of print for some time. The illustrations are beyond praise.

BOYCE, Sir R. (1911). *Yellow Fever and its Prevention*. A manual for Medical Students and Practitioners. London: John Murray, Albemarle Street, W. 380 pp., with 61 illustrations. 23 x 16 cm. Price, cloth, 10 shillings and sixpence net cash.

The volume before us presents a very attractive appearance both in the matter of printing and illustrations, many of the latter being original and of decided interest. The author has visited the chief yellow fever centres and has had an opportunity of studying the disease at first hand, commencing with the epidemic which occurred in New Orleans in 1905. In the present volume he lays stress upon the occurrence of the disease in West Africa, believing that its prevalence there has hitherto been insufficiently recognized. The book is divided into six sections dealing with: (1) the history and geographical distribution of yellow fever in Central and South America, the West Indies, South America, Europe, on Ships, and in West Africa. (2) Symptomatology and treatment. (3) Pathology. (4) Epidemiology. (5) Entomology. (6) Prophylaxis. A good index concludes the book, which will doubtless arouse some controversy in respect to the subject of yellow fever in West Africa which demands investigation.

BRESLER, J. (1910). *The treatment of Syphilis by the Ehrlich-Hata Remedy* (Dioxydiamido-Arsenobenzol), a compilation of the author's papers, translated by M. D. Eder. London and New York: Rebman Ltd, 129 Shaftesbury Avenue, W.C. 122 pp. 19 x 12 cm. Price 2 6 net, cloth.

This little book is compiled by the translator from the published observations of Bresler on the treatment of syphilis by "606"; it is embellished by two portraits of Ehrlich and Schaudinn respectively, and Dr Eder has appended abstracts of the more recent papers dealing with syphilis.

BROWN, W. C. (1910). *Amoebic or Tropical Dysentery, its complications and treatment*. London: John Bale, Sons & Danielsson, Ltd, 83-91, Great Titchfield Street, Oxford Street, W. 271 pp., 30 illustrations. 22 x 17 cm. Price, cloth, 7 shillings and sixpence net.

The author gives a general survey of the subject of amoebic dysentery and of other intestinal infections in man due to protozoa. The book is chiefly intended for practitioners and lays stress on the necessity of early diagnosis and on treatment. The twenty-four chapters composing the book deal with: (1-3) Introductory matter, History, Literature, Epidemiology and Geographical Distribution of Amoebic Dysentery. (4) Classification and general relations of

Protozoa to Intestinal Diseases. (5-8) The Entamoebae of the Human Intestinal Tract, their morphology, reproduction and cultivation. (9) Other Protozoan Infections of the Human Intestinal Tract. (10-11) Symptoms and Diagnosis. (12) Morbid Anatomy. (13) The Case for the Pathogenicity of *Amoeba histolytica*. (14) The causes of Amoebic Dysentery. (15-16) Hepatic abscess and other sequelae. (17) Prophylaxis. (18-19) Treatment of the acute and chronic disease. (20) Diet in the chronic disease. (21-23) Drug treatment, local medication, surgical and other methods of treatment. (24) Treatment of amoebic abscess of the liver—prophylaxis.

**BRUMPT, E.** (1910). *Précis de Parasitologie*. Paris: Masson & Co., 120 Boulevard Saint-Germain. 915 pp., 683 illustrations, 4 coloured plates. 20×13 cm. Price, cloth, 12 francs.

The first thing that strikes the reader on opening this book is the wealth of good and original illustration ; furthermore, that its pages contain a great deal of original matter. We have no hesitation in saying that the book will meet with success, for it is replete with information and abreast with recent work on parasitology.

Following upon an introduction dealing with general matters relating to parasitism, the first part of the book (683 pp.) deals with the Protozoa, parasitic worms, leeches, Nemathelminthes and Arthropods. The second part relates to vegetable parasites, exclusive of Bacteria ; it contains a general introduction and chapters relating to the Phycomycetes, Ascomycetes and Hyphomycetes respectively and to the diseases they produce. A good index concludes the volume, which is excellently printed and bound, and is very convenient in point of size.

**CLEMESHA, W. W.** (1910). *Sewage Disposal in the Tropics*. Calcutta : Thacker, Spink & Co. London : W. Thacker & Co., 2 Creed Lane. 232 pp., 18 figures, and tables. 22×14 cm. Price 15/- net, cloth.

This book is intended for the use of Sanitary Officers, District Engineers and others. It concerns itself mainly with septic tanks, which have been put on their trial in Bengal and have been found to work satisfactorily under Eastern conditions. The author summarizes the results of practical and experimental observations carried on in the course of the last five years. The eighteen chapters comprising the book deal with designs of latrines and tanks which have been found suitable in Bengal ; with the study of the chemical action in the tank ; the optimum rest in the tank ; analysis of sludge and gases given off ; aerobic filters ; contact-beds ; aerobic beds (the Gouripore and Fowler experimental installations) ; advantages and disadvantages of preliminary anaerobic treatment of sewage ; the "dumping" septic tank ; use of tanks in small drainage schemes ; final disposal of effluent ; "trenching grounds" ; incineration of night-soil. This work will be welcomed by all who are interested in practical matters of sewage disposal in the tropics.

**DIEUDONNÉ, A.** (1911). *Immunität, Schutzimpfung und Serumtherapie*. Zusammenfassende Uebersicht über die Immunitätslehre. 7th ed. Leipzig : Verlag von Johann Ambrosius Barth, 243 pp., few figures in text. Unbound 6.80 marks ; bound 7.80 marks.

The continued demand for a short work dealing in a trustworthy manner with

modern developments in the study of immunity has resulted in this work going through a rapid series of editions, this, the seventh, being in every way up to the character we have described in earlier notices. The book can be thoroughly recommended to all desiring to inform themselves on the subject of immunity. The additions to the present edition relate especially to Wassermann's reaction in the serum diagnosis of syphilis, to Anaphylaxis, to Chemotherapy and treatment with antiferment-sera.

DOANE, R. W. (1910). *Insects and Disease*, a popular account of the way in which insects may spread or cause some of our common diseases. (American Nature Series.) London : Constable & Company, Ltd. 227 pp., 112 illustrations. 21×14 cm. Cloth.

The contents of this popular treatise are indicated in the title. The various chapters deal with : (1) Parasitism and Disease. (2) Bacteria and Protozoa. (3) Ticks and Mites. (4) How insects cause or carry Disease. (5) House-flies or Typhoid-flies. (6) Mosquitoes. (7) Mosquitoes and Malaria. (8) Mosquitoes and Yellow Fever. (9) Fleas and Plague. (10) Other Diseases, mostly tropical, known or thought to be transmitted by Insects. The volume concludes with a useful bibliography and index. The author is Assistant Professor of Entomology in the Leland Stanford University, California, and treats the subject more satisfactorily from the entomological than from the pathological aspect. The feature of the book, which is excellently printed and illustrated, consists in the large number of original figures, mostly reproductions of photographs of disease-bearing insects and ticks.

GLAISTER, J. (1910). *A Text-book of Public Health*. Second Edition. Edinburgh : E. and S. Livingstone, 15 Teviot Place. 622 pp., 133 illustrations. 22×14 cm. Price 12/- net, cloth.

The author, who is Professor of Forensic Medicine and Public Health in the University of Glasgow, has condensed a very large amount into the covers of this book. This, the second edition of the work, differs considerably from the first edition, one of the new features being the considerable space devoted to sanitary law. The 18 chapters into which the subject-matter is divided are devoted to a sketch of sanitary progress and legislation ; vital statistics ; meteorology ; air ; heating and lighting ; water ; drainage ; sewage-disposal ; law concerning sewerage ; preventable diseases ; modes of infection and prevention etc. ; disinfection ; sanitary law in relation to infective diseases ; isolation hospitals ; law as to nuisances and foods ; diseases of occupations ; concluding with appendices relating to the Anthrax Order, 1910, and the disposal of the dead.

GUIART, J. (1911). *Les Parasites inoculateurs de maladies*. Paris : E. Flammarion, 26 Rue Racine. 362 pp., 107 text-figures, 18×12 cm. Price, unbound, 3.50 francs.

A popular treatise dealing with the part played by insects, ticks and worms in the transmission of infective diseases. Pp. 185–292 are devoted to parasitic worms and the part they may play in causing lesions which permit of bacterial invasion. The author takes the standpoint that worms play an important part in the etiology of appendicitis, typhoid, summer diarrhoea, cholera, dysentery and probably intestinal tuberculosis—in other words, according to Guiart,

worms cause infection through the lesions they produce in the intestine, in an analogous manner to the ectoparasites which by their bites introduce infection agents through the skin. "Croyez-moi," he concludes, "la vermine de l'intestin ne vaut pas mieux que celle de la peau."

JAMES, S. P., and LISTON, W. G. (1911). *A monograph of the Anopheline Mosquitoes of India*. Second Edition. Calcutta: Thacker, Spink & Co. 128 pp., 17 plates, 15 coloured plates and text-figs. 28 x 22 cm. Cloth.

The first edition of this excellent treatise having been rapidly exhausted, the authors delayed the publication of a second edition until they were able to include therein the results of their further studies upon the Anophelinae of India. The new matter includes "*a redescription of every species that was mentioned in the first edition*." A chapter is devoted to Theobald's method of classification and identification of mosquitoes by means of their scale structure. Workers on mosquitoes will have to refer to the work, since it is one of leading importance. The book presents the same general appearance as it did in the first edition, being excellently printed and well illustrated.

KINNICKUTT, L. P., WINSLOW, C. E. A., and PRATT, R. W. (1910). *Sewage Disposal*. New York: John Wiley & Sons. London: Chapman & Hall. xxvi + 436 pp., 113 figures. 23 x 15 cm. Price 12/6 net. Cloth.

The authors of this work, who are authorities on the subject of which they write, aim to discuss, somewhat fully, the fundamental principles of chemistry, bacteriology and of the engineering works in relation to sewage disposal. The book is intended for students of sanitary engineering who propose to specialize in this branch and for chemists, bacteriologists and public health officials concerned in the operation of disposal works. The 15 chapters of the work, following upon the introduction, deal with the composition of sewage ; its disposal by dilution ; screening and straining of sewage ; preliminary treatment by sedimentation, chemical precipitation and the septic process ; disposal of sludge ; purification by broad irrigation and sewage farming, by intermittent filtration through sand, by contact beds, by trickling or percolating beds ; disinfection and analysis of sewage and sewage effluents.

The work is succinctly and well written, and will prove of great use to those who are engaged in the practical work of sewage disposal. It is well printed and well illustrated.

LATHAM, A., and GARLAND, C. H. (1911). *The Conquest of Consumption*. An economic study. 2nd edition. London: T. Fisher Unwin, Adelphi Terrace. 159 pp. 19 x 12 cm. Cloth.

The second edition of this little book has followed rapidly upon the first (Jan. 1910), proving that the public is taking an active interest in the campaign against tuberculosis. The book is intended for the general reader and it contains much sound information condensed into its pages. The various chapters deal with : the cost in life ; our knowledge of the disease ; our present defences ; working-class organisation ; the cost in money (to Friendly Societies, Poor Law and Charitable Institutions, in "Life-Capital" and Wages) ; state intervention ; an efficient campaign. Some appendices refer to results achieved by working-class societies ; cheap sanatoriums ; mortality tables for tuberculosis in Wales 1900—1909. The authors lay stress upon the "urgent necessity of

Government inquiry and State intervention." The book presents an attractive appearance and it is well written.

LEON, N. (1910). *Studii asupra Culicidelor din România*. Bucharest : Institut de Arte Grafice Carol Göbl. 16, Strada Doamnei. 274 pp., 111 text-figures, 15 plates.

This publication embodies a great deal of original matter and it is to be hoped that a translation will be made so as to render it possible for those who do not master Roumanian to become familiar with the contents. The book is divided into seven chapters dealing with (1) the terminology and classification of the families Simuliidae, Psychodidae, Chironomidae and Culicidae. (2) Morphology: external and internal anatomy. (3) Bionomics etc. (4) Classification and description of species. (5) Malarial parasites and their relation to Anophelines. (6) Technique for the examination, dissection of mosquitoes and the preparation of specimens of malarial parasites therein. (7) Prophylaxis of Malaria. An appendix relates to legislative measures directed against malaria etc. and the work concludes with a good bibliography and index. The bulk of the illustrations are original, being mostly photomicrographs. They are very good.

MACEWEN, H. A. (1910). *The Public Milk Supply*. London : Blackie & Sons, Ltd, 50 Old Bailey, E.C. 182 pp., 36 figs. Price 2 shillings and sixpence net.

A book designed for the use of Medical Officers of Health, Sanitary Inspectors and others who are responsible for the administrative control of the milk supply. The author, who is evidently fully familiar with the practical aspect of dairy work, treats of the subject under five headings : (1) Milk as an Article of Commerce. (2) Milk in Relation to Disease. (3) The Housing of Animals. (4) Law Relating to the Milk Trade. (5) Dairy Products and Margarine. An appendix dealing with Cereals, Beverages, Condiments, etc. which at first sight appears out of place, is intended for the use of students working for Food Inspectors certificates whilst using another book, entitled *Food Inspection*, by the same author. The book promises to be very useful ; it is well printed and illustrated, and should meet with a ready sale in view of its moderate price.

McFARLAND, J. (1910). *Biology* : General and Medical. Philadelphia and London : W. B. Saunders Company. 440 pp., 160 illustrations. 21×14 cm. Price 7/6 net, cloth.

This book is intended for general readers and students proceeding to the study of medicine. It gives a good review of the elements of biology and differs from other books on the subject in that it treats of certain biological problems which are generally termed "medical": infection, immunity and parasitism. The 18 chapters composing the book relate to : (1) The chemical relations of living matter. (2, 3) The origin and criteria of life. (4) The manifestations of life (irritability, conductivity, motion, metabolism, reproduction). (5, 6) The cell and cell division. (7) Higher organisms. (8) Reproduction. (9) Ontogenesis. (10) Conformity to type. (11) Divergence. (12, 13) Structural and blood relationship. (14, 15) Parasitism, infection and immunity. (16) Mutilation and regeneration. (17) Grafting. (18) Senescence, decadence, and

death. The author has succeeded in compressing a great amount of information into a small space, avoiding, as much as possible, the use of technical terms. References are appended to the various chapters. The printing is good and figures instructive. Altogether the book is one to be strongly recommended.

NATTAN-LARRIER, L. (1910). *Expose des titres et des travaux scientifiques du Dr L. Nattan-Larrier.* Paris : Imprimerie de la Cour d'Appel, 1 Rue Cassette, 1. 222 pp., 57 figs.

A useful summary of the work of this talented author preceded by a list of 150 of his scientific publications dealing with the general pathology of infections and the special pathology of different organs.

PORTER, C. (1910). *Sanitary Law in Question and Answer.* For the use of Students of Public Health. London : Longmans, Green & Co., 39 Paternoster Row, E.C. 150 pp. Price 2 shillings and sixpence net.

The title and subtitle of this little book fully describe its character and the purpose for which it was written.

REINHARDT, C. (1910). *Diet and the Maximum Duration of Life.* London : The London Publicity Company, Ltd, 379 Strand, W.C. 108 pp. Price 1 shilling net, cloth.

A little treatise written in a popular vein and dealing with diet and its influence, real or supposed, upon longevity.

Ross, H. C. (1910). *Induced Cell-reproduction and Cancer.* The isolation of the chemical causes of normal and augmented, asymmetrical human cell-division, being the results of researches carried out by the author with the assistance of J. W. Cropper. London : John Murray, Albemarle Street, W. 291 pp., 125 illustrations. 23×15 cm. Price, cloth, 12 shillings net.

As stated in the introduction, "The objects of this book are to describe in detail the results obtained by a new method of experimentation with individual living human cells, their importance in the elucidation of the phenomena of healing and in the causation of cancer and other growths." The author has observed the behaviour of the blood elements *in vitro* by placing them upon a film of agar jelly impregnated with various substances whose effect upon the cell he desired to study. The results of these experiments are of considerable interest and will be found described in detail in the work. The author has studied the diffusion of various substances into cells and determined the "coefficient of diffusion" under different conditions, he has found that certain substances excite the movement of leucocytes (certain alkaloids, cancer plasma), whereas others induce them to undergo division (aniline dyes, cancer plasma etc.), the latter being termed auxetics. Blood serum was found to inhibit the action of auxetics, and, acting on the assumption that auxetics are the cause of cell-proliferation in cancer, the author has attempted to treat cases with defibrinated blood, which, according to the hypothesis, should act as an anti-auxetic. Many cases of chronic ulcers of the skin have been treated with auxetics (powdered globin and kreatin), and the results have been encouraging. A drawback to the book is the diffuse manner in which it is written—a matter which should be remedied in case a second edition is contemplated. The work is illustrated, almost throughout, with photomicrographs, the preparation of which must have entailed much labour. Many of the views expressed in the book are new and suggestive.

Ross, R. (1910). *The Prevention of Malaria* (with contributions by various authors). London : John Murray, Albemarle Street, W. 669 pp., many illustrations. 23 x 15 cm. Price, cloth, 21 shillings net.

The work before us consists of two parts exclusive of the Addenda. The first part, composed of 6 chapters, is from the pen of Ronald Ross, the second consists of contributions by various authors. The first six chapters relate to : (1) The history of malaria from ancient times to the present day. (2) Summary of facts relating to malaria (suitable for public instruction). (3) Fundamental observations and experiments. (4) The parasitic invasion in man. (5) Malaria in the community. (6) Prevention. What we have referred to as the second part consists of special contributions relating to malaria in different countries; the authors being L. O. Howard (United States), Gorgas (Panama), Le Prince (Panama), Sir Rubert Boyce (W. Indies), Prout (Jamaica), W. Thomas (Amazon Region), Oswaldo Cruz (Brazil), MacDonald (Spain), Celli (Italy), Savas (Greece), Schilling (German Possessions), Sergent (French Possessions), H. C. Ross (Egypt), Balfour (Khartoum), Bostock (S. Africa), Murison (Durban), Watson (Malay States), Takaki (Japan), R. Ross (Other Countries), Melville (Troops in War), and Fowler (Troops in Peace).

The book contains a great deal of important and new matter and will have to be consulted by all who are interested in malaria and its prevention. Numerous excellent figures illustrate the text.

THOMSON, J. A. (1910). *Outlines of Zoology*. Fifth edition. Edinburgh, Glasgow and London : Henry Frowde and Hodder and Stoughton. 855 pp. 420 illustrations. 19 x 12 cm. Cloth.

This work has already met with success, for it has reached its fifth edition. It is intended as a manual for the use of students of Zoology, being succinctly written and excellently illustrated ; most of the figures are original or drawn from good sources.

WASSERMANN, A. von (1910). *Hämolsine, Zytotoxine und Präzipitine*. Neu bearbeitet und ergänzt von J. Leuchs und M. Wassermann. Leipzig : Verlag von J. Ambrosius Barth. 124 pp. Price, unbound, 4.80 marks ; bound, 5.60 marks.

This book is based on a treatise, bearing the same title, which appeared in Volkmann's *Sammlung klinischer Vorträge* (1902), the latter having been thoroughly revised and brought up to date. It is intended for the use of medical men, as an introduction to the study of Immunity and Serology, and lays stress on the practical aspect of the experimental methods now in vogue in work on haemolysins, cytotoxins and precipitins. Needless to say, the book will find many readers.

WILEY, H. W. (1911). *Foods and their adulteration*. Origin, manufacture, and composition of food products ; infants' and invalids' foods ; detection of common adulterations, and Food standards. London : J. and A. Churchill, 7 Great Marlborough Street. 641 pp., 87 illustrations and 11 coloured plates. 24 x 16 cm. Price 21/- net. Cloth.

The present edition has been entirely rewritten in places and the part relating to the Food and Drugs Act replaced by a more extended treatment of the subject of infants' and invalids' food and to the methods of making simple tests for ordinary adulterations "which may be practised with some degree of

success in the household." The article on invalids' foods has been written in the light of recent advances in our knowledge of the subject. The subject-matter is divided into 11 parts, dealing with meats and meat products; poultry, eggs and game birds; fish foods; milk and milk products and oleomargarine; cereal foods; vegetables, condiments, fruits; vegetable oils and fats, and nuts; fungi as foods; sugar, syrup, confectionery, and honey; miscellaneous; infants' and invalids' foods; simple methods for detecting food adulterations: and an Appendix relates to food standards.

The well established reputation of the author, coupled with the importance of the subject, of which he writes in a masterly manner, should secure a wide circle of readers for the work, which is excellently printed and illustrated.

### BROCHURES.

DARLING, S. T. (1910). *Studies in Relation to Malaria*. Issued by the Isthmian Canal Commission, Laboratory of the Board of Health, Department of Sanitation. Washington : Government Printing Office. 38 pp.

Relates to the transmission and prevention of malaria in the Panama Canal Zone: Anophelines (description, infection experiments with, biology, destruction, list of species); effect of wire screening; latent malaria; effect of quinine on the parasites in man and mosquito.

GLOGNER, M. (1910). *Die Ätiologie der Beriberi und die Stellung dieser Krankheit im nosologischen System*. Leipzig : Verlag von J. Ambrosius Barth. 77 pp., 14 figures. Price, unbound, 2.50 marks.

The author, a retired "Regierungsarzt," formerly resident in the Dutch-Indies, has written this brochure with the object of proving that beri-beri is not a disease *sui generis* and that it is not due to a specific cause. Dr Glogner collected the materials for this publication during a 15 years' sojourn in Malaya where he saw a great deal of beri-beri. He considers that progress in our knowledge of the disease has been retarded by the widely prevalent view that the disease is due to a specific cause and deprecates the growing tendency to regard beri-beri as a disease-complex due no longer to one but to several as yet undetermined specific agents, thus increasing the confusion. He regards beri-beri as identical, both anatomically and clinically, with peripheral neuritis as seen in Europe; the clinical differences which are stated to exist being insufficient to permit of beri-beri being recognized as a distinct disease. It is a "Nach-krankheit."

PEDLEY, R. D. (1910). *The care of the teeth during school life*. (Issued by the Medical Officers of Schools Association.) London : J. and A. Churchill, 7 Great Marlborough Street, W. 15 pp., 6 figures. Price 1 shilling net.

(An address delivered before the Association.)

STODDART, F. W. (1911). Nitrification and the Absorption Theory. An account of the principles of the modern sewage filter. Bristol : John Wright & Sons, Ltd, Printers, Stone Bridge. 24 pp. (Printed for private circulation only.)

TELFORD, E. D. (1910). *The Problem of the Crippled School-Child*. An account of the education and treatment of crippled school-children in residential schools. London : Sherratt & Hughes. Manchester : 34 Cross Street. 32 pp. Price 6d.

## NEW JOURNALS.

**LA TUBERCOLOSI**, Vol. 1. Nos. 1—2 (1910—1911).

The official Journal of the Organising Committee of the International Congress against Tuberculosis, of which Guido Bacchelli is President and V. Ascoli Secretary General. Published in English, French, German and Italian. Rome : published by direction of the National Institute of Pharmacology in Rome.

**PALUDISM**, being the Transactions of the Committee for the study of Malaria in India. Simla : Government Central Branch Press. No. 1 (July, 1910) 53 pp., 1 plate. No. 2 (January, 1911) 117 pp. and 1 plate.

*Paludism* is a new and important periodical publication which will be issued under the authority of the Government of India, by the Sanitary Commissioner with the Government of India, Simla, and under the editorship of Major S. P. James, I. M. S., Secretary to the Central Committee for the study of Malaria in India. The publication, which will be issued at irregular intervals, is intended primarily for workers in India ; it will contain original papers and reviews. Communications should be addressed to the Editor, Simla, India. The work of the Central Committee will consist in

(A) *Routine work* : investigation on the distribution of Anophelines in India ; the formation of a type collection of mosquitoes and the identification of species sent by collectors ; studies on the biology of mosquitoes and on the prevalence of the different malaria parasites etc.

(B) *Work in the field and laboratory*, directed to the study of the conditions which determine the presence or absence of malaria in certain localities, of the mode of transmission of malaria etc. etc. Part I. contains Appendices I. and II. containing respectively questions relating to the investigation of quinine and a memorandum of questions to which answers are desired by the Central Committee ; a short syllabus of the work done at the Malaria class held in Amritsar in 1910 ; suggestions on the use of available statistics for studying Malaria in India. Notes on mosquitoes by S. P. James, being a description of a new Anopheline (*Christopersia halli* n. gen. n. sp., with plate by S. R. Christophers) ; the generic position of the Anophelines *stephensi*, *willmori* and *rossi* ; the prothoracic lobes or patagia of anophelines. Reviews of Indian and foreign Malaria literature.

No. 2 contains an editorial on the work and organisation of the committee ; Epidemic Malaria, with a note on a method of predicting epidemic years, by S. R. Christophers ; Quinine and its salts, their solubility and absorbability, by A. C. MacGilchrist ; the relation of tetanus to the hypodermic or intramuscular injection of quinine, by Sir D. Semple ; *Nyssomyzomyia rossi* and Malaria by C. A. Bentley ; the seasonal malarial infection of *Nescellia stephensi* in Bombay, by C. A. Bentley. Notes on mosquitoes (some new sp.) by S. R. Christophers ; the development of the egg-follicle in Anophelines (1 plate), by S. R. Christophers ; reviews.

**THE CHILD**. A monthly journal devoted to child welfare. Edited by T. N. Kelnack, M.D. Vol. 1. No. 1 (x. 1910). 108 pp. Single copies, price 2/- net, or 50 cents. Annual subscription, £1. 1s. 0d. or \$5.25. London : John Bale, Sons & Danielsson, Ltd.

The object of this new periodical is "to provide an authoritative and representative journal for the collection and interpretation of all subjects relating to child life." *The Child* is to be essentially a medico-sociological journal devoted to the study and protection of childhood. The first number contains short introductory articles by Sir Landen Brunton, Prof. Griesbach, Dr A. Matthieu, Prof. G. Stanley Hall, Prof. J. A. Green, Prof. M. C. Schuyten and Dr L. Dufestel; a letter from the president of the Local Government Board followed by the circular issued by the Board in relation to Poor Law Children. Excellent articles follow: the child in Germany, by Emilia V. Kanthack de Voss; education and child's brain power, by Theo. Bulkeley Hyslop; schoolgames and athletics (illustrated), by L. F. Hammer. Under the general heading of Child Problems there follow a series of short contributions upon Co-education by Sir James H. Voxall, Mrs Ennis Richmond, J. L. Patton, J. H. Badley, J. Russell and C. Grant. There follow an article on Froebel (with portrait), by Miss Temple Orme; The Leys School, Cambridge (illustrated), by Dr W. T. A. Barber; the aims and methods of the National Children's Home and Orphanage (illustrated), by Dr A. E. Gregory; the feeding of infants and children, by Dr H. T. Ashby, and minor articles followed by reviews of publications dealing with child life, correspondence etc. etc. Judging from the character of the contributors, many of whom are of international reputation, and from the subject matter presented in the opening number, this periodical is certain to meet with success provided it can maintain the standard of the first number. It will surely appeal to a wide circle of readers.

**THE MEDICAL OFFICER.** A journal for medical men in the Government and Municipal Services. Edited by A. G. R. Foulerton, F.R.C.S. Vol. v. No. 1 (7 Jan. 1911). *Supplement: The Review of Bacteriology and Parasitology.* Vol. i. No. 1, 10 pp. 30 x 22 cm. London: Hodgetts, Ltd, 36 Whitefriars St, Fleet St, E.C. Price, single numbers, 3 pence.

The publication of the *Bacteriological Supplement*, in connection with this journal, marks a new departure which will certainly be welcomed by British bacteriologists. The supplement contains a review of bacteriology and general parasitology, being an epitome of recent literature on these and allied subjects in their relation to pathology and hygiene. The supplement will be very helpful to those who have hitherto been obliged to refer to foreign journals, like the *Centralblatt für Bakteriologie* and the *Bulletin de l'Institut Pasteur*, for the information which the supplement will give. The supplement will appear under the able editorship of Dr A. G. R. Foulerton. The pages will be so numbered that they can be separately bound and indexed if desired. The first number of the supplement contains 34 reviews.

#### REPORTS.

**Annual summary of observations made at the Meteorological Station, Davos, 1909.**

Printed as supplement to the Monthly Weather Charts published by the Davos Curverein. Printed by Carl Neweczerzal, Davos-Platz.

**BANNERMAN, W. B. (1910).** *Report of the Bombay Bacteriological Laboratory for the year 1909.* Bombay: Printed at the Government Central Press. 19 pp. 34 x 21 cm. Price 5 annas or 6 pence.

- [Cairo] *Annual report for 1909. Department of Public Health.* Ministry of Interior. Paper No. 3, 1910. Cairo : National Printing Department. 131 pp., 6 figs. 33×22 cm.
- DAVIES, D. S. (1910). [Bristol City and County.] *Annual report of the Medical Officer of Health and of the General Medical Superintendent of the City Hospitals.* Special report on Smallpox. Special report on Typhoid Fever. Bristol : Bennet Brothers Ltd, Printers, Counterslip. 210 pp.
- Eighth Annual Report (1909—1910). *Imperial Cancer Research Fund.* 19 pp. 34×22 cm. London : Taylor & Francis, Red Lion Court, Fleet St.
- FREMANTLE, F. E. (1910). [Hertfordshire.] *Second annual report on School-Health in the Public Elementary Schools in Hertfordshire for the year 1909.* 95 pp.
- HAY, M. (1909). City of Aberdeen. *Report by the Medical Officer of Health for the year 1909.* With appendix on Tuberculosis in Aberdeen. Aberdeen : 112 pp.
- HILL, E. (1910). [Colony of Natal.] *Report of the Health Officer for the year ended 31st December, 1909.* Pietermaritzburg : Times Printing & Publishing Co., Ltd. 53 pp. 32×24 cm. Price 1 shilling.
- HOPE, J. W. (1910). [Western Australia.] *Annual Report for 1909 on Medical, Health, Factories, Early Closing, Vaccination, and Quarantine.* Perth : F. W. Smith, Government Printer. 64 pp. 33×21 cm.
- MERCK, E. (1910). *Annual report of recent advances in Pharmaceutical Chemistry and Therapeutics.* 1909, Volume XXIII. E. Merck, 16 Jewry Street, London, E.C. 381 pp. The book may be had free on application to the author, or may be obtainable through booksellers at 1.6 per copy.
- Ministero de Fomento. Dirección de Salubridad Pública. *Informe presentado por el Director de Salubridad Pública al Consejo Superior de Higiene sobre las medidas que deben adoptarse para preventir la importación del Córrea.* Lima : Imprenta "La Industria," Desamparados 165. 28 pp.
- NATHAN, R., THORNHILL, H. B., and ROGERS, L. (1910). *Report on the measures taken against Malaria in the Lahore (Mian Mir) Cantonment.* Calcutta : Superintendent of Government Printing, India. 55+iv pp., 1 map. 33×21 cm.
- PURDY, J. S. (1910). [Tasmania.] *Annual report for the year 1909-10.* Department of Public Health, Tasmania. Hobart : John Vail, Government Printer. 16 pp., 1 plate. 33×21 cm.
- Report of the International Commission on the control of Bovine Tuberculosis, 1910 (1911).* Department of Agriculture (Health of Animals Branch), Canada. Ottawa : Government Printing Bureau, 29 pp.
- [Report on] *Infantile Paralysis in Massachusetts in 1909* (1910). Reprinted from the *Monthly Bull. Massachusetts State Board of Health* for June, 1910. Boston : Wright & Potter Printing Co., State Printers, 18 Post Office Square. Contains the following papers : The Occurrence of Infantile Paralysis in Massachusetts in 1909, by R. W. Lovett. Infantile Paralysis as observed in Health District No. 15 during 1909, by L. A. Jones. Methods of Treatment in Infantile Paralysis, by E. H. Bradford, R. W. Lovett, E. G. Brackett, A. Thorndike, R. Souter, R. B. Osgood. The Diagnosis of Infantile Paralysis in the Prodromal and Early Acute State, as found in the Experimental Study of

Acute Poliomyelitis in Monkeys. With report of Findings in four Human Cases, by W. P. Lucas.

*Report (1911) on the IX. International Tuberculosis Conference Brussels, 6th-8th October, 1910.* Edited by Prof. Pannwitz. Berlin-Charlottenburg: Internationale Vereinigung gegen die Tuberkulose. 552 pp.

République Française (Gouvernement Général de l'Algérie 1910). *Campagne Antipaludique de 1909.* 176 pp. with maps, charts and figures. Alger: Imprimerie Algérienne.

The "Campagne Antipaludique" of 1909 in Algeria is described very fully in this official publication, which contains brief reports from some 40 medical officers, both civil and military, engaged in the anti-malaria campaign in many places in Algeria, along railway lines and amongst troops on manœuvres. A section by the brothers Sergent is devoted to studies upon epidemiology and prophylaxis: breeding places of Anophelines; endemic index; anti-mosquito measures; quinine administration etc. A very active anti-malaria campaign is being conducted in Africa and the results are proving satisfactory.

République Française. Préfecture du Dépt. de la Seine. Rapport à M. le Préfet (1910). Recherches effectuées au Bureau du Casier sanitaire pendant l'année 1909 relatives à la répartition de la tuberculose et du cancer dans les maisons de Paris. Paris: Imprimerie Chaix, Rue Bergère 20. 149 pp.

The measures taken against the dark dwellings of the poorer classes in Paris have yielded most encouraging results, tuberculosis being on the decline. These measures were rendered possible through the raising of a loan of nine hundred million francs "pour la destruction des maisons tuberculeuses."

TRUDEAU, E. L. (ix. 1910). *Twenty-sixth annual report of the Adirondack Cottage Sanitarium.* 20 pp. Saranac Lake, New York.

BROWN, L. (1910). Report of 1910. Medical Supplement. Twenty-sixth annual report for the year ending October 31, 1910. Adirondack Cottage Sanitarium. 7 pp., many charts.

*Verwaltungsbericht des Magistrats zu Berlin für das Etatsjahr 1909.* No. 18. Bericht der Deputation für die städtischen Krankenanstalten und die öffentliche Gesundheitspflege. 23 pp., also Anlage III. 28 pp. 33×26 cm. Issued December, 1910. Berlin: W. and S. Loewenthal.

#### RESEARCH LABORATORIES.

Festschrift zur Feierlichen Eröffnung des Instituts für Hygiene und Bakteriologie am 27. August, 1910. Published by Professor W. Kolle in *Arbeit. a. d. Institut zur Erforschung der Infektionskr. in Bern u. d. wissenschaftl. Laboratorien d. Schweizer Serum- und Impf-Instituts.* Heft 6. Jena: Gustav Fischer. This publication contains the following papers.

Das Institut für Hygiene und Bakteriologie (Institut zur Erforschung der Infektionskrankheiten) der Universität Bern. Plates I. and II., 17 figs., by W. Kolle. Ueber den Einfluss des Bakteriologischen Instituts der Universität Bern auf die Fortschritte der Chirurgie, by E. Tavel. Die Verbreitung und Bekämpfung der Hundswut in der Schweiz während der letzten 10 Jahre und die

Ergebnisse der Schutzimpfung nach Berichten der Pasteurabteilung. 2 figs., by O. Heller and M. Rothermundt. Die Lymphgewinnungsanstalt der Schweizer Serum- und Impfinstituts am Institut zur Erforschung der Infektionskrankheiten. 15 figs., by E. Tomarkin and H. Carrière. Die Technik der Immunisierung grösserer Tiere und der Serumgewinnung in den Laboratorien des Schweizer Serum- und Impfinstituts. 12 figs., by W. Kolle, F. Krumbein and W. Schürmann. Heizungs- und Lüftungsanlage, Warmwasserversorgung, Kührraum und Kadaververbrennungsöfen. Plates III. and IV., 1 fig., by A. A. Beutter. Verzeichnis der Arbeiten aus dem Hygienisch-bakteriologischen Institut der Universität Bern, 1896-1910.

Massachusetts Institute of Technology (1910). *Contributions from the Sanitary Research Laboratory and Sewage Experiment Station*, Volume vi. Reprinted papers. Price 1 dollar (or 4/-). Boston, Massachusetts, U.S.A. Contains the following papers.

Introduction, by W. T. Sedgwick. On the Mills-Reincke Phenomenon and Hazen's Theorem concerning the Decrease in Mortality from Diseases other than Typhoid Fever following the Purification of Polluted Water Supplies, by W. T. Sedgwick and J. S. MacNutt. The Foundations of Prevention, by W. T. Sedgwick. A Comparative Study of Intestinal Streptococci from the Horse, the Cow, and Man, by C.-E. A. Winslow and G. T. Palmer. An Investigation of the Extent of the Bacterial Pollution of the Atmosphere by Mouth Spray, by C.-E. A. Winslow and E. A. Robinson. The Disinfection of Water and Sewage, by E. B. Phelps. Disinfection of Sewage and Sewage Effluents, by E. B. Phelps. Water Pollution and Water Purification at Jersey City, N.J., by C.-E. A. Winslow. The Field for Water Disinfection from a Sanitary Standpoint, by C.-E. A. Winslow.

NEISSER, A. (1911). Bericht über die unter finanzieller Beihilfe des Deutschen Reiches während der Jahre 1905—1909 in Batavia und Breslau ausgeführten Arbeiten zur Erforschung der Syphilis. *Arb. a. d. Kaiserl. Gesundheitsamte*, Berlin. Vol. xxxvii, 624 pp. and figs.

This imposing publication records the results of investigations carried out during the years 1905—1909 by Albert Neisser and his colleagues, working in Batavia and Breslau, upon the pathology and treatment of syphilis. Their extended researches were rendered possible through financial aid from the German Government, the Prussian Kultusministerium and the Eduard Simon Foundation in Berlin. Some chapters of the report are from the pens of Neisser's colleagues Bruck and Siebert, whilst Baermann, Halberstädter, Kaiser and von Prowazek also took an active part in the work.

Following upon an introduction comes the scientific part of the report divided into 20 sections, dealing with: (1) Primary lesions in monkeys. (2) The causes of the variation in the incubative period. (3, 4) The significance of experimental investigation and of determining the presence of spirochaetes in relation to pathology and diagnosis in man. (5) The pathology of syphilis in monkeys. (6) Generalised syphilis in all kinds of monkeys. (7) Subcutaneous, (8) intravenous, (9) intraperitoneal infection experiments etc. (10) The spirochaetes. (11) What is understood by the term constitutional syphilis. (12) Immunization experiments. (13) Therapeutical experiments. (14) Serum

diagnosis. (15) Experiments and practical suggestions in respect to personal prophylaxis. (16) Prophylactic experiments with Quinine ointments. (17) Experiments upon the transmission of syphilis to different animals. The remaining sections relate to other matters. This brief epitome of the contents of the volume before us sufficiently attests its very important character.

SERGENT, Edmond (1910). *Recherches expérimentales sur la Pathologie Algérienne* (Microbiologie, Parasitologie). Algiers: Imprimerie Typographique. J. Torrent, 5 Rue Lulli. 347 pp., and many illustrations.

This publication contains the collected papers (republished) incorporating the results of experimental researches carried out during 1902—1909 in Algeria by Edmond Sergeant and his collaborators: Etienne Sergeant, E. Trouessart, Foley, Gillot, Lenaire, Bories and Ledoux. There are four sections, of which the first deals with bacteriology: Pneumococcus immunization, beer yeast and suppuration, tropism of *Bacterium zopfi* Kurth. Section II. relates to Protozoology, the occurrence of flagellates and Spirochaeta in mosquitoes, Coccidia and Haemogregarina in reptiles, trypanosomes in the frog, haematzoa in frogs and birds and the relation of the latter to mosquitoes as bearing on Schaudinn's work, hematzoa in monkeys and bats. Section III. relates to Entomology: the mosquitoes of Algeria and their relation to malaria, new species of mosquitoes being described; mosquito destruction. Section IV. deals with trypanosomiasis in dromedaries and in Berber horses, on filarial embryos in dromedaries. Section V. relates to human pathology in Algeria: malaria, myiasis, Mediterranean fever in man and goats, *Filaria persans*, trachoma, relapsing fever and *Pediculus vestimenti*, the mode of transmission of relapsing fever.

Workers in parasitology will welcome this publication, since it contains important papers published in many different journals.

*Studies from the Rockefeller Institute for Medical Research.* Volume x. (1910). Reprints. Contains the following (57) papers.

The use of the Fermentation Tube in Intestinal Bacteriology, by C. A. Herter and A. I. Kendall. *Bacillus infantilis* (n.s.) and its Relation to Infantilism, by A. I. Kendall. Pneumothorax and Posture, by C. A. Elsberg. An Experimental Study of the Influence of Kidney Extracts and of the Serum of Animals with Renal Lesions upon the Blood Pressure, by R. M. Pearce. The Effects of Roentgen Irradiation upon the Changes in the Cell Content of the Blood and Lymph induced by Injections of Pilocarpine, by R. L. Dixon. A Chemical Study of the Brain in Healthy and Diseased Conditions, with especial reference to Dementia Praecox, by W. Koch and S. A. Mann. Intestinal Obstruction: An Outline for treatment based upon the cause of Death. A study of four hundred experimentally produced Lesions, by J. W. D. Maury. The Comparative Toxicity of the Chlorides of Magnesium, Calcium, Potassium and Sodium, by D. R. Joseph and S. J. Meltzer. The effect of certain so-called milk modifiers on the Gastric Digestion of Infants, by T. W. Clarke. The Production of Edema. An experimental study of the relative etiologic importance of Renal injury, Vascular injury and Plethora Hydremia, by R. M. Pearce. The Determination of Urea in Urines, by P. A. Levene and G. M. Meyer. Further studies on the use of the Fermentation Tube in Intestinal Bacteriology, by

A. I. Kendall. A Comparative Study of the Diplococci occurring in Epidemic Cerebro-spinal Meningitis and Posterior Basic Meningitis, by M. Wollstein. An Experimental Glomerular Lesion caused by Venom (*Crotalus adamanteus*), by R. M. Pearce. Standardization of the Antimeningitis Serum, by J. W. Jobling. Blood-Platelet and Megalokaryocyte Reactions in the Rabbit, by C. H. Bunting. Continuous Respiration without Respiratory Movements, by S. J. Meltzer and J. Auer. The Butyric Acid test for Syphilis in the Diagnosis of Metasyphilitic and other Nervous Disorders, by H. Noguchi and J. W. Moore. Data concerning the Etiology and Pathology of Hemorrhagic Necrosis of the Pancreas, by E. L. Opie and J. C. Meakins. Observations on Uricolysis, with particular reference to the Pathogenesis of "Uric Acid Infarcts" in the Kidney of the New-born, by H. G. Wells and H. J. Cooper. Enzymes of Tuberculous Exudates, by E. L. Opie and B. I. Barker. Experimental Studies on Pneumococcus Infections, by S. Strouse. On Auto-antibody Formation and Anti-hemolysis, by C. E. Simon, E. Melvin and M. Roche. Proteolytic Enzymes and Anti-enzymes of Normal and Pathological Cerebro-Spinal Fluids, by A. R. Dochez. The Leucin Fraction in Casein and Edestin, by P. A. Levene and D. D. Van Slyke. The Serodiagnosis of Syphilis, by H. Noguchi. The Leucin Fraction of Proteins, by P. A. Levene and D. D. Van Slyke. On the action of Soaps upon the Vitality and Immunizing Property of *Bacillus tuberculosis*, by H. Noguchi. The Life-saving action of Physostigmine in poisoning by Magnesium salts, by D. R. Joseph and S. J. Meltzer. The Influence of Calcium upon the pupil and the Pupillomotor Fibres of the Sympathetic Nerve, by J. Auer and S. J. Meltzer. The Present Status of the Serum Therapy of Epidemic Cerebro-Spinal Meningitis, by S. Flexner. The Destructive Effect of Shaking upon the Proteolytic Ferments, by A. O. Shaklee and S. J. Meltzer. The effect of Subminimal stimulation of the Pneumogastric Nerves upon the onset of Cardiac Rigor, by D. R. Joseph and S. J. Meltzer. The Pyrines and Purine Metabolism of the Human Fetus and Placenta, by H. G. Wells and H. J. Cooper. Some Observations on the study of the Intestinal Bacteria, by A. I. Kendall. The Transmission of Acute Poliomyelitis to Monkeys, by S. Flexner and P. A. Lewis. The Transmission of Epidemic Poliomyelitis to Monkeys. A Further Note, by S. Flexner and P. A. Lewis. The Nature of the Virus of Epidemic Poliomyelitis, by S. Flexner and P. A. Lewis. Epidemic Poliomyelitis in Monkeys. Fourth Note, by S. Flexner and P. A. Lewis. Epidemic Poliomyelitis in Monkeys. A mode of spontaneous infection, by S. Flexner and P. A. Lewis. The elimination of Total Nitrogen, Urea and Ammonia following the administration of some Aminoacids, Glycyglycin and Glycylglycin Anhydrid, by P. A. Levene and G. M. Meyer. Über die Konstitution der Thymo-nucleinsäure, by P. A. Levene and J. A. Mandel. Über das Vorkommen von Prolineylanhydrid bei der tryptischen Verdauung der Gelatine, by P. A. Levene and W. A. Beatty. Über die Inosinsäure (I. II. III. Mitteilung), by P. A. Levene and W. A. Jacobs. Bemerkungen zur Wirkung von Adrenin auf die Froschpupille, by S. J. Meltzer. Über die Hefenucleinsäure, by P. A. Levene. Über die Pentose in den Nucleinsäure (I. and II. Mitteilung), by P. A. Levene and W. A. Jacobs. Über Guanylsäure (I. Mitteilung), by P. A. Levene and W. A. Jacobs. Über die

Hefenucleinsäure, by P. A. Levene and W. A. Jacobs. Über Hefenucleinsäure. (II. Mitteilung), by P. A. Levene and W. A. Jacobs. Ueber die Beziehungen von Enzymwirkungen zu den Erscheinungen der sogenannten Komplementablenkung bei Syphilis, by W. H. Manwaring. Über die Lichtextinktion, das Gasbindungsvermögen und den Eisengehalt des menschlichen Blutfarbstoffs in normalen und krankhaften Zuständen, by E. E. Butterfield.

*The Lister Institute of Preventive Medicine, London (1909-1910).* Collected papers, No. 6. Contains the following reprinted papers.

Varieties of the Meningococcus, with Special Reference to a Comparison of Strains from Epidemic and Sporadic Sources, by J. A. Arkwright. On the Production of Antitoxin by the Injection of Filtrates of Cultures of Non-Virulent Diphtheria Bacilli, by J. A. Arkwright. The Physiological Effect of Cobalt Carbonyl Vapour, by H. W. Armit. On the Paratyphoid and "Food-poisoning" Bacilli, and on the nature and efficiency of certain Rat Viruses, by F. A. Bainbridge. A note on the Spirochaete present in Ulcerative Granuloma of the Pudenda of Australian Natives, by W. C. Bosanquet. Notes on the Choanoflagellate Genera *Sulpingoeca* and *Polyoeca* with description of *Polyoeca dumosa* sp. n., by J. S. Dunkerly. The Alcoholic Ferment of Yeast-Juice. Part IV. The Fermentation of Glucose, Mannose, and Fructose by Yeast-Juice, by A. Harden and W. J. Young. The Alcoholic Ferment of Yeast-Juice. Part V. The Function of Phosphates in Alcoholic Fermentation, by A. Harden and W. J. Young. On the Nature of the Fat contained in the Liver, Kidney, and Heart, Part II., by P. Hartley. On the Wassermann Reaction in General Paralysis of the Insane, by J. Henderson Smith and J. P. Candler. Estimation of Purine Bases in Urine, by E. L. Kennaway. Observations on the Influence of Heating upon the Nutritive Value of Milk as an Exclusive Diet for Young Animals, by J. E. Lane-Claypon. Multiplication of Bacteria and the Influence of Temperature and some other conditions thereon, by J. E. Lane-Claypon. Die Synthese der Fette im Tierkörper, by J. B. Leathes. The Phagocytosis of so-called Neutral Substances. Experiments with Hippomelanin, by J. C. G. Ledingham. Observations on the Amoebae in the Intestines of Persons suffering from Goitre in Gilgit, by R. McCarrison. Further Observations on the Differentiation of Lactose-Fermenting Bacilli, with special reference to those of Intestinal Origin, by A. T. MacConkey. Observations on the Flagellates Parasite in the Blood of Fresh-Water Fishes, by E. A. Minchin. The Structure of *Trypanosoma lewisi* in Relation to Microscopical Technique, by E. A. Minchin. Observations on certain Blood Parasites of Fishes occurring in Rovigno, by E. A. Minchin and H. M. Woodcock. The Cultural Characteristics of the Microbacillus of Acne, by E. H. Molesworth. Observations on the Evolution of Immunity in Disease, by L. Noon. The Seasonal Prevalence of *Trypanosoma lewisi* in *Mus ratus* and in *Mus decumanus* and its Relation to the Mechanism of Transmission of the Infection, by C. F. Petrie and C. R. Avari. Report on Deaths which occurred in the Zoological Gardens during 1908, by H. G. Plimmer. Further Results of the Experimental Treatment of Trypanosomiasis; being a Progress Report to a Committee of the Royal Society, by H. G. Plimmer and W. B. Fry. Studies on Ceylon Haematozoa: No. 1. The Life Cycle of *Trypanosoma vivax*, by

M. Robertson. Further Notes on a Trypanosome found in the Alimentary Tract of *Pontobdella muricata*, by M. Robertson. The Development of the Parasite of Oriental Sore in cultures, by R. Row. The Hexosephosphate formed by Yeast-Juice from Hexose and Phosphate, by W. J. Young.

### REPRINTS.

- DELÉPINE, S. (xii. 1910). Contribution to the Study of Chemical Disinfectants. *Journ. Soc. Chemical Industry* (Manchester Section). 13 pp. reprint.
- KLEIN, E. (ix. 1910). The Action of Boron Preservatives on *Bacillus coli* and Allied Microbes. *Public Health*, 8 pp. reprint (price 6 pence).
- LEWIS, F. P. (30. iv. 1910). The Prevention of Blindness. *New York Med. Journ.*, 23 pp. reprint.
- (1910). The Prevention of Blindness. *Journ. Amer. Med. Assoc.*, Vol. LV. pp. 303—307, reprint.
- LOVETT, R. W., and EMERSON, H. C. (1909). The occurrence of Infantile Paralysis in Massachusetts in 1908. *Monthly Bull. Mass. State Board of Health*. 26 pp., 2 maps.
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- PEARL, R. and SURFACE, F. M. (1911). A Biometric Study of Egg production in the Domestic Fowl. II. Seasonal Distribution of Egg production. *U. S. Dep't Agricult., Bur. Anim. Industry, Bull.* 110, Part II. pp. 81—170. Washington, U.S.A.
- WASIELEWSKI, Th. v. and HIRSCHFELD, L. (1910). Untersuchungen über Kulturamöben. *Abhandl. d. Heidelberger Akad. d. Wissenschaft*, 1 Abhandl., 31 pp., 4 pls, 5 figs. 30 × 23 cm.

WASSERMANN'S REACTION IN THE SERUM DIAGNOSIS  
OF SYPHILIS, WITH RESULTS OF MERCURIAL AND  
606 TREATMENT.

BY FLEET-SURGEON P. W. BASSETT-SMITH, C.B., R.N.,  
D.T.M. & H. (CAMB.), ETC.

*Royal Naval Hospital, Haslar.*

In this report are included a series of a thousand cases examined from Jan. 1910 to Jan. 1911, the tests being made weekly at the R. N. Hospital, Haslar. Most of the cases were under observation in the hospital when their clinical characters could be recognised; in many of the cases several tests were applied, week after week, thus making the results obtained of greater value.

The technique used has been that described on previous occasions, the same care having been exercised in making full controls before the test series were examined. In almost every case Fleming's modification of the method was also utilised because it is much more easily employed, takes less time, and requires fewer reagents, but it is however not quite so reliable, erring on the side of over-sensitiveness, whereas Wassermann's technique occasionally fails in the opposite direction. The result obtained from this total of 1000 cases is shown below:

	Cases	Positive	Negative	Percentage
Syphilis (primary)	143	106	37	74 positive
, (secondary)	371	348	23	93.5 ,
, (tertiary)	64	57	7	89 ,
Latent syphilis	54	25	29	46 ,
Parasyphilis	13	13	—	100 ,
Chancroids	219	1*	218	99.5 negative
Gonorrhoea	23	—	23	100 ,
Other diseases	113	3†	110	97.4 ,

\* The sore was seen eight weeks after infection, it is not known whether the man developed secondary symptoms later.

† One case of malignant disease of the liver, in a Maltese, with intensely bile stained serum. Two cases of acute malaria.

A great number of the primary syphilitic cases were admitted as chancroids, many had mixed infections. It is of the greatest importance in these cases to be able to make a correct diagnosis so as to start specific treatment as soon as possible.

*Special reference to negative reactions found in syphilitic cases.*

Of the 143 cases of *Primary Syphilis* 74% gave a positive reaction. Of the 37 cases in which the serum gave no indication of the disease, many were very recent and had rarely received any mercurial treatment, several showed the specific spirochaete when examined by the dark ground illumination, and almost all developed secondary symptoms in the hospital; the negative reactions then, if not before, became positive ones.

*Illustrative cases.*

(1) Admitted for chancroids, six sores present, contracted on July 12th, first seen on 25th, on Aug. 12th the reaction was negative, on the 18th it had become positive.

(2) Admitted for a sore and bubo contracted on Oct. 1st, first seen on the 21st, on Nov. 3rd, 10th and 16th the reaction was negative, on the 25th it was positive, when the rash began to appear.

(3) Admitted for chancroid which was contracted on July 14th, and first seen on August 4th, on the 14th the reaction was negative, also on Sept. 1st and 9th, becoming positive on the 16th, when the rash was also seen.

(4) Admitted for a chancroid, which was contracted on July 29th, and first seen on Aug. 4th, on the 18th the reaction was negative, but on Sept. 1st it had become positive.

Only 605 of the cases of secondary syphilis gave a negative reaction, in the remainder there had been a considerable elaboration of the immune body in response to the abundance of the infective organism and its toxin. The 23 negative cases were either very early, or they had received very much treatment and the clinical symptoms were slight.

In others, where the disease was latent, negative reactions were most common.

The intermittency of the reaction was frequently seen in those who had received courses of treatment by intra-muscular injections of mercury.

For instance, in a case giving a well-marked positive reaction, after 14 injections the positive had been replaced by a negative one, which continued for some weeks, then a positive reaction reappeared to be again rendered negative after a further course of seven injections.

This disappearance and reappearance of the reaction makes it impossible to give a definite opinion from a single examination, as to whether the patient is cured or not, a point that should be fully recognised.

*At least three negative reactions at three monthly intervals should be obtained before pronouncing a case cured.*

During the year special attention has been given to :

(1) The date *after infection* when the reaction is obtained.  
 (2) The effect of much treatment on the reaction. This was determined by the study of a series of cases in which the date of infection could be definitely stated, and in which cases it was possible to take many observations. The following table has been drawn up to show the date of the appearance of a positive reaction :

Case	Disease admitted for	Negative reaction (in days)	Positive reaction (in days)	Treatment
1	Chancroid	31	37	Nil
2	Chancre of face	28	42	1 inj.
3	Sp. i	30	45	Nil
4	Chancroid	42	49	"
5	"	28. 35. 42	49	"
6	"	13. 27	55	"
7	Sp. i	49	56	"
8	Chancroid	49	56	"
9	"	36. 42. 50	57	"
10	"	27	57	"
11	Sp. i	36	60	"
12	Chancroid	58	65	"

This shows that rarely do we obtain a distinctive reaction until some seven or eight weeks after infection, namely not until the disease has become constitutional, when the glands are markedly infected or even when secondary rashes are commencing to appear. This method of diagnosis is of comparatively little help in differentiating a local non-constitutional sore from a true syphilitic chancre in its early stages.

This is in marked contradistinction to the case in which a positive diagnosis can often be made in early non-treated sores, by demonstrating

the typical living spirochaete in a few minutes by dark ground illumination, if the proper apparatus is available, or even by means of the Chinese Ink method, which is very simple and frequently as conclusive.

The number of cases having multiple sores of a non-indurated, or only slightly indurated character, that were proved to be specific by the demonstration of the spirochaete at the time (and by their subsequent course) have very much impressed those who have been interested in this work, showing the futility of giving a positive diagnosis by any other means at this early stage.

#### EFFECT OF MERCURIAL TREATMENT.

From a total of 79 cases that had received over five mercurial injections, 62 still gave a positive reaction.

With over ten injections 35 were still positive; with 20 or more there were still nine positives, and one with 25, two with 30, one with 50 and one with 70 injections still remained positive.

Four cases that had been more or less continuously under mercurial treatment for two years, or over, still gave positive reactions.

Negative reactions were obtained in three cases after six injections, in four cases after ten, and 12 injections, in three cases after 16, 18, and 19 injections, in two cases after 20 and 24 injections, in one case after 40, 50, 64, and 100 injections respectively.

The amount of mercurial treatment generally required to bring about a constant negative reaction is large; a minimum of three courses of injections being indicated in every case.

Some patients appear to be very refractory to the drug, as shown by their clinical symptoms and by the continued presence of the positive reaction, after 25, 30, 50, and 70 injections; in these the use of 606 would appear particularly applicable.

In almost all cases treated by the mouth, without intra-muscular injections, the reaction to the Wassermann test was positive.

#### *Results obtained in cases treated with 606 injections, with the serum reactions given by them, etc.*

Two lines of investigation have been followed with regard to the action of this drug on the patients to whom it has so far been administered.

(1) The serum reaction by the complemental deviation method of Wassermann.

(2) The elimination of the drug by the kidneys.

Eight cases were under observation, all benefited markedly by the remedy; I have noted this personally and so has Staff-Surgeon Shaw, R.N., under whose care the cases have been; to him I am greatly indebted for assistance and co-operation in these examinations.

The blood for the Wassermann reactions was taken together with the usual routine weekly series, the tubes containing the serum from the 606 cases, after numbering, were mixed with the rest of those under examination, and were therefore quite unknown to me until all the readings had been taken.

As 606 is believed to exert an active destructive power upon the specific spirochaete, it is to be expected that a positive Wassermann reaction would soon be replaced by a negative one owing to the diminishing quantity of the immune body called into existence by the infecting organism. It would also appear probable, supposing the dose to be insufficient, that this action would be but little marked, as shown by the continuation of the positive reaction; or if at first apparently effective, yet if the parasitic organisms were not completely destroyed, after a time there would be a renewed growth and further production of the immune body in response, with a return to a positive reaction, indicating that a cure had not been effected, and calling for further treatment.

Following this line of reasoning, the results of the following cases are interesting, but they are too few and incomplete to justify any absolute conclusions.

#### *Cases.*

(1) *Late secondary syphilis.* Before the administration of 606 the patient had received a good deal of mercury with but little benefit to the symptoms, though his serum gave a negative reaction, this negative reaction continuing after the injection and until the man was discharged.

(2) *Severe secondary syphilis.* The injection was given on Dec. 29th; on Dec. 30th and on Jan. 5th the reaction was strongly positive, on the 12th it had been converted into a negative, but on the 19th, 26th and Feb. 2nd it became again strongly positive.

(3) *Primary and early secondary syphilis.* After admission to the hospital the patient's blood gave a positive reaction, on Jan. 3rd he received an injection; on Jan. 5th, 12th, and 19th the reaction was still positive, but on the 26th it had become converted into a negative one.

(4) *Tertiary syphilis.* This patient received an injection on Jan. 8th; on the 12th, 19th, and 26th positive reactions were obtained.

(5) *Primary and early secondary syphilis.* This patient's blood was tested on Dec. 30th giving a positive reaction, on Jan. 11th he received an injection of 606, on the 12th, 19th, 26th and Feb. 2nd the reaction was still positive.

(6) *Primary and secondary syphilis.* This patient received a first injection of 606 on Jan. 12th. When the blood was examined on Jan. 19th and 26th the reaction was strongly positive; he received a second injection, but on Feb. 2nd the reaction was still positive.

(7) *Secondary syphilis.* This man's blood was tested on Jan. 5th and 19th, the reaction being positive. On the latter date he received an injection of 606. There was still a positive reaction after seven days, but in 14 days the reaction became negative.

(8) *Primary and early secondary syphilis.* On Dec. 17th, when the primary sore alone was present, the reaction obtained was negative. Secondary symptoms developed and on Jan. 23rd he received an injection, on the 26th and Feb. 2nd the reaction was strongly positive.

These observations would indicate that the immune body remains present in the serum after the injection of 606 for at least two weeks, more often for much longer, and that probably one injection is not sufficient to cause a permanent cure of the disease; further observations are however required.

#### *The excretion of the toxic arsenious drug from the body.*

It would seem to be of the greatest importance to know definitely whether the massive dose of arsenic which has been injected is retained or eliminated from the body; this elimination occurs to a great extent through the kidneys and the metal may be detected in the urine; if the patient is still passing the arsenic out in the urine complete absorption of the drug has not taken place, a further injection of 606 if given then might cause risk of serious toxic symptoms, which danger can be avoided by testing the urine for the metal.

*Detection of arsenic in the urine.* By the biological test (Gosio's), using *Penicillium brevicaulae*, a mould which has the property of breaking down organic compounds of arsenic, and giving rise to a distinct garlic-like odour.

This is undoubtedly a very delicate test.

When the mould is grown upon a media to which a dilute solution of atoxyl or salvarsan has been added the garlic-like odour is very easily detected, but if the media (bread or potato) has added to it urine from a case under treatment, which must be sterilised, infected with the mould and incubated for a day or so, then in my hands the urinary smell produced counteracts or prevents the distinctive odour from being perceived, even in samples of urine which gave a marked quantity of the metal by the chemical test. I have therefore given up this biological method as not being practicable for examination of the urine. Abel and Bnttenberg however record that 5 m. of *Liq. arsenicalis* taken can be detected in the urine.

*Chemical analysis with Marsh's test.*

In this test, which is very easily carried out, the well-known ring of metallic arsenic is obtained in the cold part of a capillary tube, through which the heated hydrogen passes.

When the drug is injected it is often absorbed very rapidly, appearing in the urine in large quantities almost immediately after its administration. In one case the urine passed *one* hour after the injection of 606 gave a very definite deposit; in all cases the first urine passed after the injection contained arsenic, the elimination continued for about 14 days; in two cases examined daily it was detected up to the 13th and 14th day respectively.

It would therefore appear that for the prevention of the toxic effects of the drug on the patient, a period of 14 days should as a rule elapse between the administration of the first and the second injection.

*Wassermann reactions in cases treated with 606 (.6 gram).*

Case		30 Dec.	5 June	12 June	19 June	26 June	2 Feb.
1	N (29 Dec.)	N					
2	(29)	P	P	N	P	P	P
3		P (31)	P	P	P	N	
4			(P)	P	P	P	
5		P (11)	P	P	P	P (30)	P
6				(12)	P	P (26)	P
7			P		P (19)	N	
8	N				(23)	P	P

( )=date of injection.

*Arsenic in urine in cases treated with 606.*

Case	After inj.	Hours	Days												
			2	3	4	5	6	7	8	9	10	11	12	13	14
1		12+		+	+	+	+	+							
2		4+			+			+							
3		4+		+	+	+	+	+	+	+	+	+	x	x	x
4		13+													-
5		5+		+	+	+									
6		1+			+							+	-		
7		3+		+	+	+	+	+	+	+	+	+	x	x	-
8		6+				+									

+ = good evidence. x slight. - nil.

*Note.* Since this paper was written in January, treatment by salvarsan has been continuously carried out at the Royal Naval Hospital at Haslar, by intravenous instead of intramuscular injections. By the intravenous method the elimination of the arsenic is undoubtedly much more rapid, very great quantities being found in the urine ten minutes after the injection, and rarely after the fourth day.

ON THE SURVIVAL OF SPECIFIC MICROORGANISMS  
IN PUPAE AND IMAGINES OF *MUSCA DOMESTICA*  
RAISED FROM EXPERIMENTALLY INFECTED LARVAE.  
EXPERIMENTS WITH *B. TYPHOSUS*.

By J. C. G. LEDINGHAM, D.Sc., M.B., M.A.  
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IT has recently been shown by Bacot (1911), some of whose experiments I had an opportunity of confirming (see Appendix to Bacot's paper), that bacilli (*B. pyocyanus*) taken up by larvae of *Musca domestica* may survive the pupal stage and reappear in the imago.

In view of the importance of this fact, it appeared desirable in the interests of practical hygiene, to ascertain whether an organism such as *B. typhosus* was also able to adapt itself to the conditions prevailing in the larval and pupal interior where presumably it would have to contend with other organisms of a more hardy character.

For the purpose of the experiments detailed in this communication, ova of *Musca domestica* were kindly supplied to me by Mr Bacot.

*First Series of Experiments.*

Ova of *Musca domestica* were received on 5 Dec. 1910. The larvae were transferred to sterile vessels containing a layer of sterilised sand and supplied with sterilised food consisting of mashed meat, potatoes and turnips. The mash was drenched repeatedly with broth cultures of *B. typhosus*. While the larvae were still feeding, it was noted that the mash had a strongly ammoniacal smell and the question arose whether the *B. typhosus* which had been supplied so liberally to it, was the predominant organism in the mash. Examination of MacConkey plates from an emulsion of the meat showed numerous colourless colonies none of which proved to be *B. typhosus*. They all gave the

characters of an organism frequently encountered throughout this work and which I shall provisionally call *Bacillus "A"*, the chief points of difference between it and *B. typhosus*, being the formation of indole and intense production of alkali in litmus milk following an initial acidity. Pure cultures of this organism gave the characteristic ammoniacal smell given by the mash in which the larvae were feeding. *B. prodigiosus* was also present in the mash.

#### *Examination of larvae before pupation.*

Four fully grown larvae were placed in absolute alcohol for 10–15 minutes and then washed repeatedly in sterile broth. Plates prepared from the mashed-up larvae gave profuse growth of colonies of *Bacillus "A"*, and a few colonies of *B. prodigiosus*. *B. typhosus* was not recovered.

#### *Examination of pupae.*

On 12 Dec. twelve pupae were found. These were transferred to sterile sand in a sterile flask. The remaining larvae were washed several times in 2% lysol followed by sterile broth and finally transferred to another sterile bottle to pupate. For the bacteriological examination of pupae various methods of disinfecting the exterior were employed, similar to those mentioned in the Appendix to Mr Bacot's paper (p. 73) but it was found impossible to obtain sterile washings before the final mashing-up process. In any case, the plates prepared from the triturated pupae gave profuse growth of colonies of *Bacillus "A"*. *B. typhosus* was not recovered.

The direct method of examining the bacterial content of the pupal interior was then devised (see Appendix to Mr Bacot's paper) and gave the following results in a series of pupae.

#### *Pupae (one-day old) examined by direct method.*

- No. I. Sterile.
- No. II. Profuse growth consisting of *Bacillus "A"*.
- No. III. Profuse growth consisting of *Bacillus "A"* and *B. prodigiosus*.
- No. IV. Sterile.
- No. V. Sterile.
- No. VI. Only one colony on plate, viz. *Bacillus "A"*.
- No. VII. Sterile.

Pupae (two days old) examined by direct method.

- No. I. Sterile.
  - No. II. Few colonies, all *Bacillus "A."*
  - No. III. Profuse growth of colonies of *Bacillus "A."*
  - No. IV. do.
  - No. V. do. + *B. prodigiosus* + *B. pyocyanus*.
  - No. VI. Mainly *B. pyocyanus*.
  - No. VII. Profuse growth of *Bacillus "A."*
- In no case was *B. typhosus* recovered from the plates.

#### Examination of emerged Flies.

The procedure adopted in examining the adult fly was as follows: the fly after being chloroformed was immersed in absolute alcohol for 5-10 minutes and afterwards transferred to a sterile slide where the wings and legs were removed. It was then passed through the flame. The abdomen was opened with sterile needles and the intestine carefully removed and mashed up in salt solution or broth.

#### Results.

*Fly No. I.* Emerged on 19th Dec. and examined on the same day: Plates showed profuse growth of *Bacillus "A."*

Films prepared from the intestine showed enormous numbers of bacilli.

*Fly No. II.* (About 10 hrs. old. Had fed on sterile cane sugar and had probably evacuated its intestinal contents.) Plates proved sterile. Microscopically only a very few bacilli were observed in stained films.

*Fly No. III.* (24 hrs. old.) Plates sterile.

*Fly No. IV.* (24 hrs. old.) Plates showed *Bacillus "A," B. prodigiosus* and *B. pyocyanus*.

*Fly No. V.* (48 hrs. old.) Pure plates of *Bacillus "A."*

*Fly No. VI.* (do.) (do.)

*Fly No. VII.* (do.) *Bacillus "A"* and *B. pyocyanus*.

*Fly No. VIII.* (do.) One colony of *Bacillus "A."*

In all cases where the plates showed profuse growth of colourless colonies, a large number were picked off and carefully examined on the chance that the typhoid bacillus might be recovered, but in no case was a successful result obtained. All the colonies picked off proved invariably to consist of *Bacillus "A."*

*Second Series.*

Ova were received on 4th and 5th Jan. 1911 from Mr Bacot, and within 12–24 hrs. all had hatched out. On this occasion cultures were made from the egg-shells and some of the newly emerged larvae, as the negative results obtained in the first series of feeding experiments with *B. typhosus* had given rise to the suspicion that this micro-organism had only a very small chance of survival in competition with organisms contaminating the exterior of the ova and which were probably ingested at a very early period by the newly emerged larvae.

The plates showed profuse growth consisting of the following organisms, *B. proteus vulgaris*, *Bacillus "A,"* *B. prodigiosus* and a *Streptococcus*. The larvae were fed with a sterile mash and kept at 25° C. until the 9th Jan. when several full-grown individuals were found.

These were washed in carbolic solution and finally transferred to a fresh sterile vessel containing sterile sand to which a liberal supply of typhoid broth cultures had been added. One, however, was not placed in the typhoid chamber but was retained for bacteriological examination. Plates prepared from it gave a growth consisting of *Bacillus "A,"* *B. prodigiosus* and a lactose fermenter (*Bacillus "B,"* No. 3, vide Table I). On 10th Jan., one larva was removed from the typhoid chamber and examined after careful disinfection in carbolic solution. The plates consisted mainly of *Bacillus "A"* and *B. proteus vulgaris* but one colony was found to consist of *B. typhosus*. Whether this organism actually came from the larval interior could not be decided, as the broth in which the larva had been washed after disinfection gave after incubation a growth which consisted of *Bacillus "A,"* and *B. proteus vulgaris*.

*Examination of Pupae.*

Pupa No. I. (12 hrs. after pupation.) Plates gave pure growth of *Bacillus "A."*

Pupa No. II. (12–24 hrs. after pupation.) Profuse growth of *Bacillus "A."*

Pupa No. III. (3–4 days after pupation.) Plates consisted of *Bacillus "A,"* *Bacillus "C"* (see Table), and *Bacillus "E"* (see Table).

Pupa No. IV. (4 days after pupation.) Plates consisted of *Bacillus "A"* and *B. prodigiosus.*

In no case was *B. typhosus* recovered from the pupae.

One adult fly was examined after it had fed on sterile sugar for two days.

The plates proved sterile.

*Third Series.*

On 24 Jan. 1911 a new batch of eggs was received and an attempt was made to sterilise the eggs before the emergence of the larvae, but before sterilisation a sample of the eggs was taken for bacteriological examination. The organisms isolated were chiefly those marked in the Table as *Bacillus "B"* No. 3, *Bacillus "B"* No. 2, and *Bacillus "D."* The remaining eggs were placed in 5% lysol for 2½ mins., washed repeatedly in saline solution and allowed to dry as far as possible. Sterile human blood was then given them but unfortunately the eggs became entangled in the fibrin meshes and no larvae emerged.

*Final Series.*

On 25 Jan. another batch of newly emerged larvae was received from Mr Bacot. *These had developed from eggs which had been disinfected by a short sojourn in lysol.* The young larvae had been placed on a sterile agar slope which remained sterile. Human blood mixed with typhoid bacilli was spread on the agar and this process was repeated, the larvae being transferred to a fresh agar slope with blood every day.

*Examination of larva.*

On 29 Jan. one larva was removed from the slope and placed in 5% lysol for 5–10 mins. After successive baths of saline solution and absolute alcohol the larva was mashed and plated.

The plates yielded what appeared to be a pure growth of typhoid-like colonies.

Seven of these were picked off, all of which proved to be *B. typhosus*.

Films made from the mashed-up larva also showed numerous typhoid-like organisms with a few large diplococci.

*Further examination of larvae.*

On 31 Jan. four larvae were found dead in the condensation fluid of the agar, the excess not having been removed by an unfortunate omission. Only one larva survived. Two of the dead larvae were placed in 2% lysol for 10 hours and then plated.

*B. typhosus* was recovered in pure culture from each.

The surviving larva was placed on fresh sterile sand and pupated between 3rd and 4th Feb.

*Examination of pupa.*

On 6 Feb. the pupa was examined by the direct method. The colonies on the plates were few but all of them proved to be *B. typhosus*. They were agglutinated by an anti-serum in dilution of 1 in 2000.

## SUMMARY AND CONCLUSIONS.

Although typhoid bacilli were liberally supplied to larvae of *Musca domestica*, all attempts to demonstrate *B. typhosus* in the pupae or imagines were unsuccessful, until recourse was had to disinfection of the ova. After this preliminary disinfection both larvae and pupae gave pure growths of *B. typhosus* but hitherto it has not been possible to examine the imagines.

In the experiments with unsterilised ova great difficulty was experienced in determining whether *B. typhosus* was present in MacConkey plates owing to the almost invariable occurrence of the colourless typhoid-like colonies of the *Bacillus "A"* which was evidently an organism thoroughly adapted to the conditions prevailing in the interior of the larvae, pupae and imagines.

The fact also that this organism like *B. typhosus* fermented mannite without the production of gas, rendered the search for *B. typhosus* still more difficult.

By the employment of sorbite—in place of lactose-media, a preliminary differentiation might have been possible but was not considered practicable in the first place owing to the expense of sorbite, and secondly owing to the fact that the fermentation of sorbite by *B. typhosus* though constant, is sometimes delayed. The most satisfactory plan was to pick off as many colonies as possible and inoculate them on litmus-milk. *Bacillus "A"* invariably rendered litmus-milk intensely alkaline.

From the practical point of view the main conclusion to be drawn from the experiments detailed in this communication is that the typhoid bacillus can lead only a very precarious existence in the interior of larvae or pupae which possess, at least in so far as these investigations warrant, a well-defined bacterial flora of their own.

Even under the highly artificial conditions of the final series of experiments, it was not possible to decide whether the *B. typhosus* though recoverable from the pupa was really actively multiplying in the pupal interior or gradually dying out. There was some indication that the latter was the case, as the typhoid colonies recovered from the pupa

in the one successful instance, were extremely few in number, while the larvae which had been feeding on *B. typhosus* contained enormous numbers as evidenced both by cultural and microscopical examination.

Since the above experiments were concluded, some further researches by Graham-Smith (1911) on the carriage of bacteria by flies have appeared in the form of a Local Government Board Report. This author has succeeded in recovering *B. anthracis* from blow-flies bred from larvae fed on meat infected with spores of this organism, but has failed to recover *B. typhosus* or *B. enteritidis* under similar experimental conditions.

TABLE I. *Characters of the chief organisms isolated from ova, larvae, pupae and imagines.*

(*B. typhosus* inserted for comparison with *Bacillus "A."*)

	Motility	Liquefaction of gelatine	Lactose	Glucose	Mannite	Dulcite	Saccharose	Sorbitol	Litmus milk	Indole	Adon.	Inul.
<i>B. typhosus</i>	+	-	-	A	A	-	-	A	A	-		
<i>Bacillus "A"</i>	+	-	-	A	A	-	-	-	A - alk.	+		
<i>B. prot. vulg.</i>	+	+	-	AG	-	-	AG	-	AC pept.	+		
<i>B. prodigiosus</i> (α)	+	+	-	AG	A	-	A	A	AC pept.	-		
do. (β)	+	+	-	AG	A	-	AG	A	AC pept.	+		
<i>Bacillus "B" No. 1</i>	-	-	AG	AG	AG	AG	AG	AG	AC	-		
do. No. 2	+	-	AG	AG	AG	-	AG	AG	AC	-		
do. No. 3	-	-	AG	AG	AG	AG	AG	AG	AC	+	AG	-
<i>Bacillus "C"</i>	+	+	-	AG	AG	-	AG	-	Pept.	+		
<i>Bacillus "D"</i>	+	-	-	AG	AG	-	AG	AG	AC	-		
<i>Bacillus "E"</i>	-	-	-	A	-	-	-	-	A	-		

*Notes on the organisms in Table I.*

*Bacillus "A."* Compare with organism marked 4B isolated by Morgan from faeces of children (see Morgan and Ledingham, *Proc. Roy. Soc. of Med. Epidem. Sect. March, 1909*, Table I). Also isolated by the writer from faeces of typhoid convalescents and examined by Morgan (see *Journ. of Hyg.* Vol. xi., No. 1, April, 1911, Table II. Organisms B 20—B 25). They are not agglutinated by typhoid or dysentery serum.

"B" No. 1—"B" No. 3. Lactose-fermenters, belonging to Groups III and IV in MacConkey's scheme (see MacConkey, "A Contribution to the Bacteriology of Milk." *Journ. of Hyg.* Vol. vi., 1906).

*Bacillus "D."* Compare with organisms marked No. 12 and No. 13, isolated from faeces of children by Morgan (see Morgan and Ledingham, *ibidem*).

The occurrence of *B. pyocyanus* is explained by the fact that Mr Bacot had been experimenting with this organism. The ova had become contaminated by fly excreta containing this organism among others.

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## THE ACTION OF CERTAIN BACTERIA ON PROTEINS.

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THE digestion of solidified blood-serum by certain sporing bacilli is well known, and Bienstock (1899) and Rettger have shown that *B. putrefactive* and other obligate anaerobic sporing bacteria destroy protein and cause putrefactive changes. Hardly any observations have been made, however, to determine whether aerobic, non-sporing bacilli and cocci have any power to break down proteid, although it is often assumed they can do so. Rettger (1906) found that bacilli of the colon group did not set up putrefactive changes in a mixture of egg and meat protein; he made no quantitative experiments as to whether any destruction of protein took place, and Dr C. J. Martin informed me that he has for long noticed that preparations of pure proteins, when left exposed to air, did not appear to be broken down by bacteria, although moulds were able to grow in them.

In the present enquiry an attempt has been made to ascertain whether certain aerobic organisms can break down either pure proteins, or solutions of protein in the presence of various nitrogenous extractives.

### METHODS.

#### I. *Preparations of pure proteins.*

Three proteins were selected for examination, namely

- (1) Egg-albumin;
- (2) The total coagulable protein (globulin and albumin) of horse serum: and
- (3) Alkali-albumin, prepared from egg-albumin.

(1) *Egg-albumin.* Crystalline egg-albumin was prepared by Hopkins' method and dissolved in distilled water. The protein was

then precipitated with anhydrous sodium sulphate at 37° C., washed with a solution of sodium sulphate of the same sp. gr. as the filtrate, dried between sheets of blotting-paper, and redissolved in distilled water. This process was repeated many (8–10) times, until the solution was almost free from ammonia.

For the final solution, ammonia-free distilled water was used.

Most of the sodium sulphate was removed by allowing it to crystallise out at 0° C. and by subsequent dialysis of the protein solution. The solution was finally sterilised by filtration through a Berkefeld filter, previously washed with distilled water. One obtained in this way a fairly strong (2–5 %) solution of egg-albumin which served as a stock.

(2) *Serum protein.* The total coagulable protein present in normal horse serum was precipitated by the addition of anhydrous sodium sulphate, the serum being warmed to 37° C. The precipitate was washed with a solution of sodium sulphate of the same specific gravity as the filtrate, dried between sheets of blotting-paper, and redissolved in a small quantity of distilled water. This process was repeated 10–12 times until a protein solution was obtained, which was almost free from extractive nitrogen. For the final solution, ammonia-free distilled water was used; most of the sodium sulphate was removed by allowing it to crystallise out at 0° C. and by subsequent dialysis of the protein solution for an hour.

The solution, which was yellowish in colour, was finally sterilised by filtration through a Berkefeld filter and was kept as a stock.

Two or three preparations of egg-albumin and serum-protein were made in the course of the experiments, and varied somewhat as to their relative purity.

(3) *Alkali-albumin.* The material used had been prepared from pure egg-albumin by Dr C. J. Martin. It was dissolved in a 0·5 % sodium carbonate solution; normal hydrochloric acid was then added until a point was reached at which the protein began to be precipitated. The amount of free ammonia present in these solutions was estimated and used as an indication of their purity, since it is very difficult to remove all the free ammonia from such solutions by the precipitation method. The following figures were obtained for two samples:

(1) 1 e.c. of a stock solution of serum protein contained 0·000008 grm. NH<sub>3</sub>.

(2) 1 e.c. of a solution of alkali-albumin contained 0·000008 grm. NH<sub>3</sub>.

## II. Preparation of media.

For the purpose of estimating the multiplication of bacteria in solutions of pure protein, a weak solution (0·1-0·5 %) of protein was made by diluting a portion of the stock solution of protein with ammonia-free distilled water. Various salts were added and the final composition of the medium was as follows :

Protein 0·1-0·5 %.

Sodium chloride 0·5 %.

Sodium sulphate (approximately) 0·1-0·25 %.

Calcium chloride trace      }  
Potassium phosphate trace } approximately 0·1 %.

Sulphuric acid ( $\frac{N}{100}$ ) was added until the medium, which at first was distinctly alkaline, became very faintly alkaline to extremely sensitive red litmus paper. It was then sterilised by filtration through a Berkefeld filter, and put in test tubes, so that each contained roughly 5 c.c. of fluid.

For the chemical estimation of protein the media were prepared in the same way, except that stronger solutions of protein were used. Usually 20-25 c.c. of the medium were accurately measured with a sterile pipette into a number of small flasks, which after incubation at 37° C. for two days were tested as regards their sterility. Some were kept as controls, the remainder being inoculated with various organisms from fresh agar cultures. All the flasks were incubated at 37° C. for 7-28 days, the same time being allowed in each experiment. The amount of coagulable protein present in each flask was then estimated. The solution was poured into a small beaker, made distinctly acid to sensitive neutral litmus paper by the addition of  $\frac{N}{100}$  butyric acid, and boiled for several minutes until the protein was coagulated and the supernatant fluid was perfectly clear. The coagulum was collected on a weighed filter paper, dried and weighed. In one or two experiments, the residual nitrogen was estimated by Kjeldahl's method after removal of the protein by tannic acid; and occasionally the amount of free ammonia present in the solution was determined by the vacuum distillation method.

### III. Method of determining the multiplication of organisms.

The test tubes containing the media were incubated at 37° C. for 24 hours and then tested as regards their sterility. A small quantity of the organisms under examination was taken from a fresh agar culture by means of a fine platinum needle and added to the fluid in the test tubes. After half an hour, the tube was well shaken and two drops were taken from the test tube, by means of a capillary pipette, and dropped into 5 c.c. of sterile normal saline solution which was well shaken. This was called the *first dilution*. With the same pipette two drops were taken from the dilution and dropped into a second test tube also containing 5 c.c. of sterile saline solution, known as the *second dilution*. In some cases a third dilution was made in the same way. Five drops and one drop from the original medium and from each dilution were dropped into petri plates and ordinary agar plates were made, taking care to ensure a good mixture. After incubation of the plates for 24 hours, the colonies on each plate were counted. All the plating was done in duplicate. The test tubes containing the inoculated media were incubated at 37° C. and the number of organisms present was determined from day to day by the above method.

The same pipette was used throughout the whole of the experiment, and was dried each time before and after being used as a "dropper" so that the size of the drops should be, as far as possible, uniform. As a rule five organisms were examined in each experiment, and aseptic precautions were always taken.

Other details as to the methods used are given subsequently.

The micro-organisms used in the experiments were as follows :

- (1) *B. coli communis*, stock strain, Lister Institute.
- (2) *B. enteritidis* Gaertner, original strain.
- (3) *B. typhosus* Guy, stock strain, Lister Institute.
- (4) *B. proteus*, stock strain, Lister Institute.
- (5) *B. pyocyanus*, stock strain, Lister Institute.
- (6) *Staphylococcus p. aureus*, from an acute abscess.
- (7) *Gonococcus*, from case of acute gonorrhoea.

The same strains were used throughout the whole investigation.

## RESULTS.

**I. MULTIPLICATION OF BACTERIA IN MEDIA CONTAINING ONLY  
PURE PROTEIN AND SALTS.**

Visible growth rarely occurred, though a faint opalescence was occasionally observed in the case of the less pure preparations of protein.

The multiplication of the organisms (as determined by plating) was influenced by two factors, namely, the purity of the medium and the number of the organisms originally added to the medium, and the actual figures obtained in the various experiments show only a general correspondence with one another.

Even the purest preparations of the proteins contained traces of ammonia, and probably other nitrogenous extractives; and some of the organisms used, notably *B. enteritidis* Gaertner, can exist and even multiply to some extent in solutions containing the merest traces of organic material. In this enquiry it was found that, when the bacterial seeding was small, some multiplication took place in all the protein media, but when a larger bacterial seeding was made, the numbers of organisms diminished in the purest protein media, whereas some multiplication occurred in the less pure protein media.

The general results are given in Tables I-III. They show that no extensive multiplication of bacteria occurred, although the seeding was usually large, and that the organisms examined grew very little better in solutions of protein than in solutions of glucose (and salts), in ammonia-free distilled water. The power to multiply was least in the case of *B. typhosus* and *Staphylococcus aureus*. In fact cultures of these two organisms on protein media were sometimes sterile, when subcultured after incubation for a week or more.

In a number of experiments, 0·5 to 1% glucose was added to the protein media. It seemed possible that the organisms might be able to break down protein, when a readily utilisable source of energy and of carbon was present. The addition of sugar had very little influence, however, on the growth of the organisms examined, as long as the protein medium was pure. A very faintly acid reaction of the medium to sensitive red litmus paper was sometimes observed, but in other cases no difference could be detected between the inoculated and the control tubes.

Since glucose is readily attacked by all the organisms employed, when available nitrogenous food is present, the minimal destruction of glucose (as judged by the formation of acid) in these experiments seems to show not only that the media contained very little non-proteid nitrogen, but also that very little (if any) proteid was broken down.

The fact that when comparative tests were made with the same sample of a protein medium, the number of organisms tended to become constant after a few days with either a large or small seeding (Table IV), also suggests that the organisms were living on the traces of non-protein nitrogen in the medium rather than on the protein itself. In the latter case one would expect a progressive increase in the number of organisms as more and more protein was used up, whereas, in point of fact, the number of organisms tended to decrease after three or four days. When a few drops of broth or of impure solution of albumose were added to the protein medium, all the organisms showed multiplication, and *B. coli* and *B. enteritidis* grew freely, indicating that the media contained nothing which inhibited or interfered with the growth of organisms.

#### *Media containing gelatin.*

A few observations were made with a medium containing 10% gelatin, salts and distilled water; the gelatin was not purified at all. The medium was made neutral to sensitive red litmus paper. *B. proteus*, *B. pyocyanus* and *Staphylococcus aureus* grew very feebly on this medium; and although some liquefaction eventually took place, it was greatly delayed. When 1% glucose was added to the medium, *Staphylococcus aureus* showed very little growth, *B. proteus* and *B. pyocyanus* grew rather more vigorously, but did not visibly liquefy the gelatin for ten days, although they liquefied control tubes of ordinary broth gelatin very rapidly.

It is probable that a medium consisting only of gelatin, salts and water, is very slowly digested by these organisms, chiefly because they grow feebly and therefore produce very little digestive enzyme. But this may not be the only factor, since the conditions which influence the activity of the extracellular proteolytic enzymes of *B. proteus* and *B. pyocyanus* are not fully worked out; and it is not known how far an acid or alkaline reaction of the medium modifies their proteolytic power.

## II. THE ACTION OF BACTERIA UPON PROTEIN, AS DETERMINED BY CHEMICAL METHODS.

The amount of coagulable protein present in protein media before and after inoculation with various organisms was estimated by the coagulation method. One or two observations were also made to determine whether the presence of bacteria in solutions of pure bacteria and salts influenced the amount of uncoagulable nitrogenous material in the solutions.

### (a) *Pure proteins.*

When the solutions contained only pure protein and salts, none of the organisms examined were able to break down the protein. The results, given in Table V, show that the amounts of coagulable protein in the control and inoculated flasks were the same, allowing for the experimental error of the method. Even *B. proteus* and *B. pyocyanus* did not destroy protein when it was pure.

The observations as to the amount of non-coagulable nitrogenous material present in control and in inoculated flasks containing solutions of pure protein showed no evidence that any destruction of protein occurred in the inoculated flasks.

### (b) *Solutions of protein and nitrogenous extractives.*

A number of experiments were made to determine the effect of adding to solutions of pure protein, either albumoses, peptone, broth or other nitrogenous material. It seemed possible that, when sufficient material was present to ensure vigorous bacterial growth, the protein might be broken down. Chemical methods alone were available for this investigation.

The substances added to the protein solutions in different experiments were the following:—

- (1) Ordinary peptone broth in sufficient amount to form a 0·3% solution (medium A).
- (2) An impure preparation of albumose, prepared from Witte's peptone, but still containing some extractive nitrogen (medium B).
- (3) Horse serum diluted and freed from coagulable protein by boiling in acid solution. The filtrate contained no coagulable protein, but gave a very faint Xantho-proteic reaction (medium C).

(4) Horse serum freed from protein by filtration through a silica filter (Martin, C. J., 1896).

(5) *Medium E.* Diluted horse serum, heated to 55° C. for half an hour to destroy complement.

Suitable amounts of sodium chloride and other salts were added to all the media, which were then *neutralised* and filtered through a Berkefeld filter. 20 c.c. were accurately measured into a number of sterile flasks; some of these were kept as controls, others were inoculated with various organisms. After incubation for six or more days at 37° C. the total coagulable protein present was estimated.

The results are given in Tables VI and VII. They show that, in the presence of an adequate supply of nitrogenous food, *B. proteus* can break down protein, but that the other organisms examined do not disintegrate it—at any rate, to any appreciable extent. No constant diminution in the amount of coagulable protein occurred, and the small differences observed in individual experiments are due to the technical difficulties of the method.

Good growth occurred in all cases, and the reaction of the solutions became alkaline as compared with the controls. The degree of alkalinity was measured by noting the number of drops of  $\frac{N}{10}$  butyric acid required to render the solution neutral to very sensitive red litmus paper. The degree of alkalinity of the media (after incubation) varied with the different organisms used, being greatest for *B. proteus* and least for *Staphylococcus pyogenes aureus*.

Serum-proteins are fairly sensitive to acids and alkalies, and are gradually disintegrated in a strongly alkaline solution. But the amount of alkali formed by the bacteria used in these experiments was apparently too small to cause any appreciable destruction of the protein present in the media, even when the initial reaction of the medium was slightly alkaline to sensitive litmus paper. When a mixture of serum protein and peptone broth was used, none of the coagulable protein was destroyed, whether the initial reaction of the medium was neutral or slightly alkaline.

It may be noted also that proteins themselves can act either as weak acids or as weak alkalies, and that when present in large amount (as in blood serum) must exert a considerable influence in keeping the reaction of the medium near the neutral point.

This action, on the part of proteins, is probably of importance in modifying the way in which bacteria break down non-protein nitrogenous

substances, since it is well known that the activities of many organisms are closely dependent upon the reaction of the medium in which they are placed. The *Gonococcus*, for example, will grow well on ordinary agar, to which pure serum-protein or egg-albumin is added, although there is no evidence from chemical analysis to show that it can break down serum-protein. It will also grow on agar, to which is added the serum (deprived of all protein), but containing phosphates. Indeed, as Blair Martin (1910) has shown, strains of *Gonococcus* which fail to grow on ordinary agar will grow readily when phosphates are added.

Apparently, the *Gonococcus* requires for its existence and multiplication a medium of a particular degree of alkalinity, and the presence of proteins or phosphate serves to keep the reaction of the medium at a suitable point.

### III. THE FORMATION OF INDOL FROM PURE PROTEIN.

So far as is known, indol is formed by bacteria only from tryptophane or from proteins (or polypeptides) which contain a tryptophane radicle. Whether any bacteria, when growing in media free from a tryptophane radicle, can produce small traces of indol as a product of their own metabolism is not known. In the present enquiry, *B. coli* was cultivated on some media, which responded to none of the tests for a tryptophane group; no indol could be detected in these media.

It seemed, therefore, that the formation of indol in media containing only pure protein would indicate that the indol had been formed by bacteria from the protein. The media examined were:

1. Solutions of pure egg-albumin and serum-protein.
2. These solutions with the addition of 1% urea.

The media were neutral to litmus paper and were sterilised by filtration through a Berkefeld filter. A series of test tubes containing these media were inoculated with *B. proteus* or *B. coli communis*; after incubation for 5–10 days they were tested for indol by the paradiamethylamidobenzaldehyde method. A negative reaction was obtained in all cases.

It is clear from these experiments that the organisms examined do not break down appreciable amounts of pure protein, and that *B. coli*, *B. pyocyaneus*, *B. enteritidis* and *Staphylococcus aureus* do not cause any gross destruction of protein, even in the presence of an adequate supply of non-protein nitrogenous food. There is, in fact, no evidence that these organisms can break down protein at all; possibly the protein

molecule is too large to be taken up by the organisms. It is important to remember, however, that exceedingly small quantities of nitrogenous food are needed to maintain bacterial life and permit of some multiplication; and it is conceivable that the organisms examined may be able to break down very minute traces of protein, traces so minute as to escape detection by the methods employed.

#### CONCLUSIONS.

(1) When solutions of pure egg-albumin, serum-protein or alkali-albumin are inoculated with the organisms studied in this enquiry, the bacteria diminish in number when the seeding is large, but multiply to some extent when the seeding is small. The evidence goes to show that the multiplication observed with small seedings is due to the presence in the medium of traces of non-protein nitrogenous substances, and that the bacteria do not use protein as food.

(2) There is no chemical evidence that the organisms which have been studied are able to break down pure egg-albumin and serum-protein; but such very minute amounts of nitrogenous food suffice to maintain bacterial life that, with the methods available, one cannot be certain that the organisms did not disintegrate and use as food very small traces of protein.

(3) The organisms examined (with the exception of *B. proteus*) do not break down appreciable quantities of egg-albumin and serum-protein, even in the presence of sufficient non-protein nitrogenous food, to ensure vigorous bacterial growth.

I wish to acknowledge my indebtedness to Dr C. J. Martin for constant help and advice in the course of this enquiry.

TABLE I.

The organism examined was *B. typhosus*.

The figures represent the number of organisms actually counted on the plates made *in duplicate* from the various dilutions of the medium.

(a) Egg-albumin.		Period of incubation in days			
		0	1	2	6
Dilution examined		Uncountable	—	—	—
Medium itself, 5 drops					
,, 1 drop		"	—	Uncountable	—
1st dilution, 5 drops		243, 239	222, 190	626, 528	298, 310
,, 1 drop		60, 50	46, 38	201, 158	80, 75
2nd dilution, 5 drops		—	—	—	5, 1
,, 1 drop		—	—	—	1, 0
(b) Serum-protein.		Period of incubation in days			
		0	1	2	
Dilution examined					
1st dilution, 1 drop		Too many to count	405, 492	192, 212	
2nd dilution, 5 drops		609, 490	16, 18	10, 10	
,, 1 drop		55, 90	5, 3	1, 3	
3rd dilution, 5 drops		7, 21	1, 0	1, 0	
,, 1 drop		1, 1	0	0	

TABLE II.

The figures represent the number of organisms present in one drop of the medium, which was examined at the time of inoculation and on subsequent days.

In the case of most of the organisms, two experiments are recorded, one with a small seeding and one with a large seeding.

(a) Egg-albumin.		Period of incubation in days				
Organism examined		0	1	2	3	4
<i>B. typhosus</i> (1)	...	6,025	5,150	15,150	—	—
" (2)	...	56,125	—	20,375	—	14,375
<i>B. coli communis</i> (1)	...	11,125	—	—	35,500	—
" (2)	...	495,875	—	153,750	—	30,000
<i>B. enteritidis</i> (1)	...	185,625	—	245,625	—	232,500
" (2)	...	37,500	—	144,375	—	—
						8 days 26,250.
(b) Serum-protein.		Period of incubation in days				
Organism examined		0	1	2	3	4
<i>B. typhosus</i> (1)	...	1,029,375	31,875	18,750	—	—
" (2)	...	34,250	45,375	—	17,125	—
<i>B. coli communis</i> (1)	...	335,575	123,750	50,625	—	—
" (2)	...	200,000	60,125	57,500	20,250	—
<i>B. enteritidis</i>	...	468,750	288,750	264,375	—	211,875
<i>B. proteus</i>	...	840,000	520,000	393,750	—	—
<i>Staphylococcus aureus</i> (1)	2,148,750	1,312,500	161,250	—	—	
" (2)	228,750	157,500	86,250	—	—	
(c) Alkali-albumin.		Period of incubation in days				
Organism examined		0	1	3	6	
<i>B. typhosus</i> ...	...	150,000	59,250	32,875	28,875	
<i>B. coli communis</i> ...	...	143,750	—	153,750	112,500	
<i>B. enteritidis</i> ...	...	843,750	—	453,750	61,875	
<i>B. proteus</i> ...	...	71,000	—	150,000	60,000	
<i>Staphylococcus aureus</i> (1)	90,000	59,415	56,250	—		
" " (2)	52,250	—	29,375	2,000		
(d) Serum-protein + 1% glucose.		Period of incubation in days				
Organism examined		0	2	4	15	
<i>B. coli communis</i> ...	...	843,750	423,125	153,750	—	15 days. No indol.
<i>B. enteritidis</i> ...	...	1,350,000	1,025,625	468,750	900 (approx.)	
<i>B. proteus</i> ...	...	1,428,750	480,000	69,375	—	15 days. No indol.
<i>Staphylococcus aureus</i> ...	...	300,000	205,500	41,500	—	
(e) Alkali-albumin + 1% glucose.		Period of incubation in days				
Organism examined		0	2			
<i>B. coli communis</i> ...	...	77,000	98,500	5 days, slightly acid to litmus.		
<i>B. typhosus</i> ...	...	613,125	570,000	5 days, neutral to litmus.		
<i>Staphylococcus aureus</i> ...	...	1,076,250	973,125			

TABLE III.

The organism used was *B. coli communis*.  
 The figures represent the number of organisms present in one drop of the medium at the time of inoculation and on subsequent days.

Medium used	Period of incubation in days				
	0	1	2	3	4
(1) Normal saline solution	12,300	—	—	425	—
(2) 1% glucose + salts	33,875	221,250	—	119,250	—
(3) Egg-albumin (a)	... 11,125	—	—	35,750	—
(4) " (b)	... 496,875	—	—	153,750	—
(5) Serum-protein (a)	... 330,000	123,750	48,700	—	—
(6) " (b)	... 71,375	55,125	—	30,625	—
(7) Serum-prot. + glucose	843,750	—	423,125	—	153,750
(8) Alkali-albumin	... 143,750	—	153,750	—	—
(9) Alk.-alb.+glucose	... 20,000	—	250,000	—	—

TABLE IV.

The medium used was pure serum-protein. The organism examined was *B. coli communis*. The figures represent the number of organisms actually counted on the plates.

## I. Small seeding.

Dilution counted	Period of incubation in days		
	0	1	3
1st dilution, 1 drop	13	1212	810
2nd dilution, 5 drops	1	69	61
" 1 drop	1	10	12

## II. Large seeding.

1st dilution, 1 drop	1168	1000 (approx.)	1015	9 days. No indol.
2nd dilution, 5 drops	66	97	103	
" 1 drop	10	25	28	

TABLE V.

*The action of bacteria upon solutions of pure protein and salts.*

In all cases the initial reaction of the medium was as nearly as possible neutral. Control experiments were always made.

The figures represent the weight of coagulable protein in grammes present in the various flasks. Each flask contained 20 c.c. of the protein solution.

The period of incubation varied from 8-24 days.

Organism examined	Serum-protein		Egg-albumin	
	1+glucose	2	1	2
Control (1) ...	0.0942*	0.1097	0.1488	0.0944
, (2) ...	0.093	0.1115	0.1500	0.0944
<i>B. coli communis</i> ...	0.1008	0.1084	0.1470	—
<i>B. enteritidis</i> Gaertner	0.0963	0.1084	0.1455	—
<i>B. proteus</i> ...	0.1010	—	0.1480	—
<i>B. pyocyanus</i> ...	0.0992	0.1083	0.1492	0.0964
<i>Staphylococcus aureus</i>	0.1013	0.1076*	0.1507	—
<i>Gonococcus</i> ...	0.0972	—	—	—
Period of Incubation	12 days	24 days	14 days	8 days

\* Slight loss of protein.

TABLE VI.

*The action of bacteria on solutions containing serum-protein, salts, and various non-coagulable nitrogenous substances.*

In all cases the initial reaction of the medium was as nearly as possible neutral. Control experiments were always made.

The figures indicate the weight of coagulable protein present in the various flasks. The period of incubation varied from 9-28 days.

Each flask contained 20 c.c. of the solution.

Organism examined	A	A	C	C	E	
	Alkaline	Protein + peptone-broth neutral	Serum-prot. +filtr.+salts	1	2	1
Control (1) ...	0.0886	0.1470*	0.0780	0.1109	0.1884	0.3563
, (2) ...	0.0860	0.1519	0.0776	Lost	0.1806	0.3507
<i>B. coli communis</i> ...	—	0.1554	0.0778	—	0.1710	0.3485
<i>B. enteritidis</i> Gaertner	0.0948	0.1569	0.0792	0.1120	0.1758	0.3169*
<i>B. pyocyanus</i> ...	0.0906	0.1565	0.0542	0.1130	0.1640	0.3558
<i>B. proteus</i> ...	0.0527	—	0.0295	—	0.1322	0.2986
<i>Staphylococcus aureus</i>	—	—	0.0738	—	0.1906	—
<i>Gonococcus</i> ...	—	—	—	0.1102	—	—
Period of Incubation	—	9 days	10 days	17 days	9 days	28 days

\* Slight loss of protein.

TABLE VII.

*The action of bacteria on solutions containing egg-albumin, salts and various non-coagulable nitrogenous substances.*

In all cases the initial reaction of the medium was as nearly as possible neutral. Control experiments were always made.

The figures indicate the weight of coagulable protein in grammes present in the various flasks. The period of incubation varied from 6-10 days.

Each flask contained 20 c.c. of the solution.

Organism examined	A Egg-albumin + peptone-broth	B Egg-albumin + albumose	C Egg-albumin + filtered-serum
Control (1) ...	0·1923	0·1209	0·1392
" (2) ...	0·1902	0·1162	0·1406
<i>B. coli communis</i> ...	0·1968	0·1210	0·1419
<i>B. enteritidis</i> Gaertner	0·1918	0·1199	0·1396
<i>B. pyocyanus</i> ...	0·1861	0·1200	0·1592
<i>B. proteus</i> ...	—	—	0·1042
<i>Staphylococcus aureus</i> ...	0·1938	0·1188	—
Period of Incubation	6 days	6 days	10 days

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## AN OUTBREAK OF DYSENTERY.

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*(Medical Officer of Health for Paddington.)*I. *History of the outbreak.*

ON February 10th, 1911, the attention of the Medical Officer of Health was called by the practitioner in charge to an outbreak of diarrhoea in a family named R. residing in Paddington. The note made at the interview was to the effect that cases had occurred on the 8th, 9th, and 10th (2 cases) of the month, the four patients having violent diarrhoea and passing much blood. The onset was quite sudden in every case. It was thought that "food poisoning" could be excluded, and the main object in directing attention to the outbreak was to ascertain if any defective drainage existed at the house. Incidentally it may be remarked that the house drainage, etc. had been completely overhauled in 1907 and that no defects existed in February last except one leaky joint on a waste pipe.

Inquiries were at once set on foot and it was found that the cases of diarrhoea were more numerous than stated.

The house is an old one, originally a good class dwelling, one of a group of houses known to one of us since the '60's when they were occupied by well-to-do families. The house at the time of the inquiries was let out in six tenements, the occupants numbering 30.

Cases of diarrhoea were found to have occurred in the R. and H. families who were much together (Mr R. keeps a coffee-stall and Mr H. assists him). No history of any attacks could be obtained among the other occupants of the house.

Half basement (2 rooms) and 2nd floor (attic) front (1 room);	R. family; man, wife and 10 children, ages 16 yrs.—3 mths., twins.
Ground floor (2 rooms) and 2nd floor (1 room);	L. family, 3 adults and 4 children.
First floor back (1 room);	H. family, 2 adults and 3 children.
First floor front (1 room), and 2nd floor (1 room);	X. family, 3 adults.
First floor (1 room);	One adult (away).
Second floor front (1 room);	Two adults (away).

A further case was found in a family named J. residing in the neighbourhood, the patient being a cousin of the R. children and much with them.

Unfortunately, as so frequently occurs, rather more than a fortnight had elapsed between the date of the first attack and the commencement of the inquiry (February 11th). Numerous visits of inquiry elicited the following history.

(1) R. R., f. aet. 5, taken ill January 26th with sickness, severe abdominal pain, much diarrhoea with blood. She was seen by a Dr C. on the next day and was well again on the 29th.

(2) E. R., f. aet. 5, taken ill with same symptoms on the 29th. She was seen by Dr C. and appears to have recovered by February 5th.

(3) C. R., f. aet. 7, was attacked about 3rd February. She had no sickness and was still ill on the 11th.

(4) W. J., f. aet. 3, attack of same kind commencing on February 4th; attended St Mary's Hospital as out-patient.

(5) A. R., m. aet. 2. Attack commenced February 5th. His attack, one of the severest of the series, was attended with collapse. He was still under treatment on the 11th.

(6) B. H., f. aet. 1, bad diarrhoea on February 5th (no medical attention).

(7) E. R., m. aet. 3 months,—one of twins—was taken ill on the 8th or 9th.

In addition to the seven well-defined attacks there were two others in which the history was less definite.

I. R., f. 11, slight diarrhoea ( $\frac{1}{2}$  day) on February 5th.

T. R., m. 14, "ailing" from about February 10th. No history of diarrhoea could be obtained.

Of the R. children, three escaped attacks, viz.  
J. R. (m. 16), J. R. (m. 10) and the other twin.

None of the parents in the R., H. or J. families were ill.

The fact that the cases occurred very nearly singly and in succession may be taken as conclusive evidence that "food poisoning" was not the causal factor. Further, the fact that all the children who were ill were closely associated—almost living together—while other children living in the house but not associating with the R., H., or J. families, escaped, points to personal infection, or transfer of infection through common use of closet, etc.

## II. *Bacteriological examination.*

1. *Faeces and urine.* On February 14th, 1911, samples of faeces and urine from two patients (E. and R. R.), and faeces only from a third patient (L. H.) were received. The material was plated out on lactose-neutral-red-bile-salt agar, and the white colonies were picked off, and examined as regards their morphological and cultural reactions. The faeces and urine of one patient (E. R.) yielded a negative result, as did also the urine of R. R. The faeces of R. R. and L. H., however, yielded numerous colonies of non-motile bacilli which had the following characters:

1. Production of acid on glucose, mannite, sorbite, arabinose, galactose and laevulose.
2. No visible fermentation of lactose, cane sugar, dulcrite, adonite or inulin, salicin, raffinose, maltose, xylose or glycerin.
3. Production of indol.
4. Milk showed initial acidity, but became alkaline in fourteen days.

On February 21st samples of faeces were received from L. H., E. R., R. R., T. R., L. A. R., and W. J.; all these children had suffered from clinical symptoms of dysentery. The faeces were soft and shiny in some cases, hard and green in others. On examination, bacilli identical with those first described were obtained from the faeces of T. R. and E. R.; the others gave a negative result.

Finally on February 25th and March 1st samples of faeces were obtained from the parents of the R. family and from Mr H. They had not been ill, and no dysentery bacilli were found in their stools.

The agglutination reactions of the organisms are shown in Table I. All the strains were tested with sera obtained from rabbits immunised

with *B. dysenteriae* (Flexner), *Bacillus "Y"* of Hiss and Russell, and *B. typhosus* respectively. It will be seen that the strains were not agglutinated by the Flexner serum in such high dilutions as *B. dysenteriae* (Flexner) or the "Y" bacillus, but they were agglutinated by the "Y" serum to the same extent as *B. dysenteriae* (Flexner) and *Bacillus "Y."* They were agglutinated in low dilutions only by the typhoid serum. These results correspond with those obtained by Morgan (1911) in the case of British strains of *B. dysenteriae*.

TABLE I.

Agglutination tests. Macroscopic method.

Organisms examined	<i>B. "Y"</i> serum	<i>B. Flexner</i> serum	<i>B. typhosus</i> serum titre 4,000
<i>B. dysenteriae</i> , Flexner	2,000	5,000	—
<i>Bacillus "Y"</i>	10,000	5,000	—
L. H.	1,000	1,000	800
E. R.	1,000	1,000	—
T. R.	1,000	1,000	—
R. R.	1,000	1,000	100

Absorption tests were not made as they seem to be of little value for the differentiation of this group of bacilli.

2. *Samples of blood.* Blood was obtained from three patients, namely R. R., T. R., and L. H., and tested as regards its agglutinating power for *B. typhosus* and *B. dysenteriae* (Flexner). The results are given in Table II.

TABLE II. Agglutinating power of patients' serum.

Microscopic method. Controls were used in each case.

Patient	Organism tested	
	<i>B. typhosus</i>	<i>B. dysenteriae</i> (Flexner)
R. R.	Positive reaction	Positive reaction (1-100)
T. R.	Negative ..	.. ..
L. H.	" "	" "

In the case of R. R. the reaction was more complete with *B. dysenteriae* than with *B. typhosus*.

Sera, prepared by injecting *B. dysenteriae* into rabbits, usually contain secondary agglutinins for *B. typhosus*, so that the reaction of the serum of R. R. with both typhoid and dysentery is not surprising.

The presence of *B. dysenteriae* in the faeces of the patients, and the strong agglutination of this bacillus by the serum of three patients,

furnish evidence that this bacillus was the cause of their illness. Apart from Asylum dysentery, outbreaks of dysentery in this country appear to have been very rarely observed; the only instance previously recorded was a fatal case occurring in a child in London, in which Marshall (1909) obtained from the faeces a bacillus identical in cultural and agglutination reactions with *B. dysenteriae* (Flexner).

Morgan (1911), however, has recently examined a large number of strains of dysentery bacilli, which had been isolated by Ledingham from the faeces of typhoid convalescents and from other sources, and his observations seem to indicate not only that dysentery bacilli of the Flexner type are more often present in the faeces of healthy persons than has been hitherto supposed, but also that slight differences in the fermentation reactions of different strains are of minor importance.

On receiving information of the results of the bacteriological examination, inquiries were made to ascertain if any members of the affected families had been abroad or whether there had been any contact with persons recently arrived from foreign parts. Neither Mr nor Mrs R. had ever been out of the country, nor had any visitors from abroad. Mr H. had served in the Royal Army Medical Corps, being stationed at the Cambridge Hospital, Aldershot, where patients from all parts of the world are received. He had, however, left the Army some ten years ago. His faeces, moreover, were free from the *B. dysenteriae*.

It is worth noting that positive results were obtained twice from the first patient (E. R.), an interval of one week intervening between the taking of the specimens; also that T. R., in whose faeces the *B. dysenteriae* (Flexner) was found, and whose blood gave a positive reaction with the same bacillus, was not ill to the same severity as were other children from whose faeces the bacillus could not be isolated. T. R.'s trifling attack occurred ten days prior to the day on which his specimen was taken.

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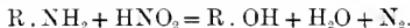
THE ACTION ON NITRATES AND NITRITES OF DYSENTERY  
ORGANISMS KILLED BY VARIOUS PROCESSES AND  
OF FILTRATES FROM FLUID CULTURES.

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IT has been shown previously (Logie 1909 and 1910) that the power of reducing nitrates to nitrites is possessed by the majority of organisms belonging to the dysentery group. Thus of 15 different dysentery strains, examined by the author, only one failed to reduce nitrate. This strain did not ferment glucose, mannite, maltose, or saccharose. In the case of these dysentery bacilli which ferment mannite (Flexner, Strong and Y types) the reducing action proceeds further and the nitrite finally disappears. On the other hand, *B. dysenteriae* Shiga which rapidly reduces nitrate to nitrite does not carry the reduction further, even when the cultures are kept for many weeks in the incubator. A number of factors have been found to modify the reducing action of bacteria. Free access of oxygen diminishes the reducing action (Burri and Stutzer 1895, Weissenberg 1897, Logie 1910). Thus if equal amounts of inoculated fluid medium be distributed (*a*) in conical flasks so as to form a thin layer and expose a large surface to the air, and (*b*) in ordinary test tubes, reduction proceeds much more rapidly in the latter. Indeed by filling the flask with oxygen reduction may be quite prevented. On the other hand, anaerobic conditions (*e.g.* filling the flasks or test tubes with hydrogen) do not cause reduction of nitrite by *B. dysenteriae* Shiga, or the other organisms which do not usually reduce nitrite. The presence of glucose does cause *B. dysenteriae* Shiga to

destroy nitrite, but this is due to the formation of acid from glucose which leads to the liberation of free nitrous acid, which in turn liberates the amide nitrogen of the peptone according to the equation



The following experiments were undertaken with a view to the further elucidation of the nature of the reducing power of dysentery bacilli. It was necessary to determine whether (1) the bacilli formed some soluble substance which possessed the reducing action (a reductase) or (2) the reducing power was inseparable from the organism and (3) if in the latter case dead bacilli exerted this action.

#### *The action of filtrates.*

For this purpose *B. dysenteriae* Flexner and *B. dysenteriae* Celli were selected, as these strains were found to be very active reducers of nitrite. The action of *B. coli* was also tested.

Small Jena glass flasks each containing 100 c.c. of peptone water (1%) were inoculated with these organisms, and incubated for 24 to 48 hours at 37° C. The cultures were then sucked through small Maassen filters of unglazed porcelain (measuring externally 3·5 in. by 0·6 in.) into filter flasks by means of a water pump. With a sterile pipette the filtrate was distributed in amounts of 5 c.c. in test tubes. To each test tube was added ·025 c.c. of a  $\frac{n}{600}$  solution of sodium nitrite. A similar amount of sodium nitrite was added to 5 c.c. of sterile water, the latter being used as a colour control. Some of the tubes of filtrate were reinoculated from agar slope cultures of the homologous organism. Two other controls were used, consisting each of 5 c.c. of the original unfiltered culture. To the one was added ·025 c.c. of an  $\frac{n}{600}$  solution of nitrite. To the other a similar amount of  $\frac{n}{600}$  nitrate solution. The tubes were then sealed and incubated at 37° C. for from five to 14 days. At the end of that period the tubes were opened, subcultures made, and the presence or absence of nitrite ascertained by means of the test with  $\alpha$ -naphthylamine acetate and sulphanilic acid. The results were as follows:—

Filtrate	With nitrate	With nitrite
Filtrate reinoculated	Slight colour	Nitrite reaction
Original fluid culture	" "	" "
	No colour	No reaction

The control series to which no addition of nitrite or nitrate had been made gave no reaction with the test. The slight nitrite reaction in the tubes containing nitrate seemed to be due to the difficulty of obtaining a nitrite-free solution. It will be noted that the tubes of filtrate reinoculated from agar cultures of the homologous organism behaved very much like the filtrate alone, while the tubes of unfiltered fluid culture show complete reduction both of nitrate and of nitrite. No reduction by the filtrate could be detected even when it still contained some organisms. In the reinoculated tubes of filtrate, growth tends to be very scanty, and this no doubt accounts for the difference between these tubes and those containing unfiltered culture. Thus it appears that these bacilli do not produce a reducing substance which diffuses out into the surrounding medium to any marked degree. It is known that in the process of filtration substances present in solution may be retained to some extent by the substance of the filter. Serum complement and rennin may be thus retained, but as Muir and Browning (1909) have shown for serum complement, such action tends to occur only at first, the filter latterly becoming permeable. In the above experiments the amount of fluid passed through the filter was considerable in proportion to the size of the latter, and it is most unlikely that in this instance the whole of the reducing substance has been retained by the filtrate. The conclusion therefore seems to be justified that no soluble reducing substance is produced.

In studying the effect of dead organisms experiments were made with organisms killed by heat, by certain antiseptics and by acetone.

#### *The action of organisms killed by heat.*

In the case of organisms killed by heat the following method was employed. Cultures were made on agar of *B. dysenteriae* Flexner, *B. coli*, and *Staphylococcus aureus*, and after 24 hours incubation at 37° C. these were made into emulsions with sterile water. The emulsions were considerably denser (at least three or four times) than bouillon cultures of the same organism after 24 hours at 37° C. and contained 750 to 1500 million organisms per 1 c.c. As it was desirable that nitrate or nitrite from the agar or the condensation water should not be carried over into the emulsion, the cultures were removed by means of a platinum loop, and carefully rubbed down on the sides of the tubes containing the sterile water. The emulsions were made as homogeneous as possible. The emulsion having been prepared the

sides of the test tubes above the emulsion were heated in the bunsen flame till all the material on the side of the tube was carbonised. The tube was then plugged with sterile cotton wool, and immersed in a water-bath at 56° C. Cultures were made about every ten minutes, and it was found possible to sterilise four emulsions at a time, the subcultures being made in rotation from each. As a rule, cultures taken after heating for one hour at 56° C. were quite sterile, but in one case when *B. coli* and *B. dysenteriae* Flexner were kept at 54° C. all the cultures gave growths.

The subcultures were made by taking loopfuls of the emulsion and smearing them on sloped agar, and it was possible by this means to trace the gradual diminution in the number of living organisms. Thus in one experiment with *B. coli* the subcultures gave the following result after 24 hours at 37° C.

Cultures taken after	Result after incubation for 24 hours at 37° C.
16 mins.	Copious growth.
26 "	Fairly abundant growth. Numerous confluent colonies.
36 "	31 colonies, of 0·5 mm. to 3 mm. diam.
46 "	Only four colonies. One of 1 mm. and three of 2·5 mm. diam.
56 "	No growth.
66 "	" "
80 "	" "

In this case the temperature of 53° C. to 55° C. had been maintained for one hour and 20 minutes. It is to be noted that a marked degree of inhibition is possible with ultimate growth. Whether this is an indication that different individuals in the cultures possess very variable degrees of resistance to heat, must be left undecided (v. H. Chick 1910). Thus in one experiment a subculture which showed only three colonies of *B. dysenteriae* Flexner after 24 hours at 37° C. showed six after 48 hours, and nine after some days, while one which seemed sterile the first day had developed one or two colonies a few days later.

It was thus important that more than one subculture should be sterile, before deciding that the culture had been killed, and it was always necessary to test the sterility at the end of the experiment before applying the test for nitrite. On one occasion a *Staphylococcus aureus* emulsion which had given no growth either on the last three controls or on a control agar slope inoculated from the dense emulsion after it had been incubated at 37° C. for 24 hours, gave after a fortnight's incubation quite a copious growth of *Staphylococcus aureus*, when a small loopful was smeared on agar and incubated at 37° C. for 24 hours.

The nitrite solution which had been added was sterile. This capacity of organisms so delicate even as those of the dysentery group to revive after a somewhat lengthy period of inhibition, is a point which must be borne in mind in all experiments where it is necessary that sterilisation should be effected with a minimal amount of disturbance of function, as for instance in obtaining endo-enzymes or vaccines. It seems that this is an important source of possible fallacy, especially as organisms may revive to some extent during the experiment, and yet perhaps die off before the end.

The emulsions having been made and sterilised as above described, an amount of nitrite enough to make an  $\frac{n}{60,000}$  solution was added, and the tube sealed to exclude air, as it has been shown that in the course of incubation a considerable amount of nitrate may be absorbed from the air. A similar tube of sterile water containing the same amount of nitrite was sealed as a control, and the tubes were incubated for several days or even weeks. At the end of the period of incubation the tubes were opened, subcultures made and the presence or absence of nitrite ascertained by the usual test. In this way it was found that reduction might apparently take place with dead cultures of *B. dysenteriae* Flexner and *B. coli*. In three experiments out of 15, reduction was obtained, but the amount of reduction was very slight when compared with that obtained with living fluid cultures, containing considerably fewer organisms, even when the latter had been growing for several days before nitrite was added. In the other 12 experiments no reduction was detected even after incubating for a week at 37° C. In none of the experiments was a growth of organism obtained from the heated emulsion, either at the beginning or at the end of the experiments. But the possibility cannot be excluded that in the positive experiment some of the bacilli at least had retained for a time a slight degree of vitality. Furthermore the degree of nitrite reaction obtained was so slight as to suggest that it might be due to some experimental error. Thus the dead bacilli have little if any action in reducing nitrates and nitrites.

#### *The action of organisms killed by antiseptics.*

The action on nitrites and nitrates of organisms killed by antiseptics was also studied, five antiseptics being used, viz. sodium fluoride, tetrachlor-o-biphenol, tetrabrom-o-kresol, p-chlor-o-kresol and hexabrom-dioxy-diphenyl-carbinol. The four latter, for which we are indebted to the kindness of Professor Ehrlich, are antiseptics of high value, whose

action on *B. diphtheriae* and some other organisms has been studied by Bechhold and Ehrlich (1906). Briefly it may be stated that a 1% solution of tetrachlor-o-biphenol ( $\text{OH} \cdot \text{Cl}_2\text{C}_6\text{H}_2 - \text{C}_6\text{H}_2\text{Cl}_2 \cdot \text{OH}$ ) poured over a 24 hour agar culture kills *B. diphtheriae* in less than two minutes and *B. coli* in less than five, while it inhibits the growth of *B. diphtheriae* in bouillon culture in a strength of  $\frac{1}{400000}$ . A 1% solution of phenol does not, even when allowed to act for 60 minutes, completely kill an agar culture of *B. coli*. Similarly a 1% solution of tetrabrom-o-kresol ( $\text{C}_6\text{Br}_4 \cdot \text{CH}_3 \cdot \text{OH}$ ) kills an agar culture of *B. diphtheriae* in less than two minutes and of *B. coli* in less than five, while in the strength of  $\frac{1}{200000}$  to  $\frac{1}{100000}$  it inhibits the growth of *B. diphtheriae* in bouillon. A 1% solution of hexabrom-dioxy-diphenyl-carbinol



kills *B. diphtheriae* (agar culture) in less than two minutes, but a 3% solution takes over an hour to kill *B. coli*. It must be remembered that the molecule of hexabrom-dioxy-diphenyl-carbinol weighs twice as much as the molecule of tetrachlor-o-biphenol and more than seven times that of phenol, so that strictly speaking a 7.3% solution of hexabrom-dioxy-diphenyl-carbinol corresponds molecularly to a 1% solution of phenol.

Taking phenol as a standard and measuring the power of inhibiting the growth of *B. diphtheriae*, Bechhold and Ehrlich found that 0.7 of a molecule of tetrachlor-o-biphenol, 0.9 of a molecule of tetrabrom-o-kresol, and 0.6 of a molecule of hexabrom-dioxy-diphenyl-carbinol were each equivalent to 1000 molecules of phenol. The theoretical interest of this lies in the relationship established between the chemical composition and the antiseptic power. The introduction of the halogen atoms (Cl, Br, etc.) or the uniting of two phenol groups either directly or by means of a CHOH group increases the antiseptic power of the molecule.

The *B. dysenteriae* Flexner emulsions used by the author contained so many organisms that they can hardly be used for comparison with Ehrlich and Bechhold's results. It was found, however, that tetrachlor-o-biphenol, tetrabrom-o-kresol and p-chlor-m-kresol completely killed the emulsions in 24 hours at 37° C. in a strength of  $\frac{1}{5000}$  to  $\frac{1}{10000}$ , while hexabrom-dioxy-diphenyl-carbinol required a strength of  $\frac{1}{4000}$  to sterilise the emulsion and sodium fluoride a 2% solution. On one occasion a dense emulsion of *B. dysenteriae* Flexner in a saturated NaF solution (4-5%) gave copious growths from platinum loopfuls smeared on agar even after 12 hours and 24 hours incubation of the emulsion. A dense

emulsion of *B. dysenteriae* Flexner in normal saline solution was killed in 30 hours by a  $\frac{1}{2000}$  dilution of hexabrom-dioxy-diphenyl-carbinol, and by a  $\frac{1}{15000}$  dilution of p-chlor-m-kresol. It was also killed in the same time by a  $\frac{1}{500}$  solution of phenol, but not by  $\frac{1}{1000}$  solution. For the dysentery group also, phenol is therefore a much less efficient antiseptic.

The organisms were grown on  $\frac{1}{2}\%$  agar in large Kolle flasks and transferred by means of a platinum scraper to 0·85% salt solution in a strong sterile test tube. The latter contained a quantity of glass beads, by means of which the culture could be broken up and thoroughly emulsified by shaking. In practice it was found that even better results were obtained by using a fairly wide test tube and raking the beads about by means of the scraper. The emulsion always contained from five to ten millions of bacteria; that is to say, many times the number which occurs in any peptone water or bouillon culture. It was, indeed, quite thick and creamy, so that objects were invisible through a test tube containing it, whereas type can be made out through a bouillon culture.

Of each of the phenol derivatives, 0·1 grm. was dissolved with the least possible amount of NaOH solution and the volume was made up to 100 c.c. with sterile water, and diminishing amounts of this 0·1% solution were added to a series of sterile 4 in. by  $\frac{1}{2}$  in. test tubes, the bulk being made up in each case to 1 c.c. with sterile salt solution. One cubic centimetre of bacterial emulsion was then added to each tube and the tubes were sealed with paraffin. After 24 hours incubation the tubes were opened, subcultures taken and 0·2 c.c. of sodium nitrate solution ( $\frac{n}{500}$ ) added. The subcultures were made by withdrawing 0·2 c.c. of the contents of the test tube and adding it to an amount of peptone water sufficiently large to make the antiseptic ineffective. With the amounts of antiseptic used, 5 c.c. of peptone water is sufficient, but in some experiments 200 c.c. were employed. The nitrate having been added, the tubes were sealed and incubated for from three to ten days. They were then reopened, subcultures again made, and the test with  $\alpha$ -naphthylamine acetate and sulphanilic acid applied for nitrites. The following is an account of such an experiment:

The growth from six 5 in. by  $5\frac{1}{2}$  in. Kolle flasks was made into an emulsion with 40 c.c. salt solution. Six series of tubes were prepared, series (a) containing tetrachlor-o-biphenol, series (b) tetrabrom-o-kresol, and series (c) p-chlor-m-kresol in dilutions of  $\frac{1}{5000}$ ,  $\frac{1}{10000}$ ,  $\frac{1}{20000}$ ,  $\frac{1}{40000}$ . Series (d) contained hexabrom-dioxy-diphenyl-carbinol in dilutions of  $\frac{1}{2000}$ ,  $\frac{1}{4000}$ ,  $\frac{1}{10000}$ ,  $\frac{1}{20000}$ , and series (e) sodium fluoride in 2%, 1%, 0·5%,

and 0·04% solution. To each tube was added 1 c.c. of *B. dysenteriae* Flexner emulsion. Four controls (5 a, 5 b, 5 c and 5 d) containing bacilli but no antiseptic were also put up.

The sixth series (*f*) consisted of five tubes each containing one of the antiseptics in the same amount as the first tube of its series, and to this series autoclaved emulsion (120° C. for 15 minutes) was added. A sixth tube of each set contained the same amount of antiseptic as the first tube, but no bacilli, the volume being made up to 2 c.c. with salt solution. Each tube therefore contained 2 c.c. of fluid. After incubation for 24 hours the tubes were opened, subcultures made and sodium nitrate added to make an  $\frac{n}{5000}$  solution. The tubes were sealed and incubated for ten days. They were then opened and tested for nitrite. The results of the various tests are comprised in the table on p. 369.

It will be noted that of the salt solution controls (tubes 6 ag, 6 bg, 6 cg, 6 dg, and 6 gg), two (6 ag and 6 dg) show traces of nitrite. This may be due to some slight reducing action of the antiseptic or to solution of nitrite from the glass of the test tube during the prolonged incubation; it might also result from some imperfection in the sealing of the tube which allowed absorption from the air to occur. This very slight colour, however, is of quite a different order from the marked reaction given by tubes 3 and 4 of series (*a*), 3 and 4 of series (*b*), 2, 3 and 4 of series (*c*), 3 and 4 of series (*d*), or 2 and 3 of series (*e*).

The slight trace of nitrite found in the first tube of each series and in all the tubes of the 6th (*f*) series, may be due to nitrite carried into the emulsion with the bacilli or to one of the causes suggested above in the case of the salt solution control. It is of the same order as the traces found in the salt solution control and must be considered an experimental error. In this connection one must remember the extreme delicacy of the  $\alpha$ -naphthylamine reaction. Studying each series by itself, we find that first we have tubes which show only a trace of nitrite and give no growth in the sterility control. Then come one or two tubes which give a very marked nitrite reaction, indicating reduction of the nitrate. These all give growth when tested by culture. Finally in the tube containing no antiseptic, the reaction varies from a distinct colour to nothing at all. In these the reduction of nitrate to nitrite has occurred just as in peptone water or bouillon culture and the nitrite has subsequently been reduced. It thus appears that a series of tubes containing diminishing amounts of antiseptic give very similar results to a series of sample tubes taken at intervals

from a peptone water or bouillon culture. The first tubes give no reaction owing to non-reduction of the nitrate. The later tubes give a strong reaction, owing to the reduction of nitrate to nitrite,

Antiseptic	No. of tube	Amount of antiseptic	Sterility		Result of test for nitrite
			before nitrate added	at end of experiment	
Tetrachlor-o-biphenol	1 a	5 1/5	—	—	Trace
	2 a	1 0 1/00	—	—	" "
	3 a	2 0 1/00	G	G	Very marked
	4 a	2 0 1/00	G	G	" "
	5 a	None	G	G	Very faint trace
	6 ag	5 1/5	—	—	Trace
Tetrabrom-o-kresol	1 b	5 1/5	—	—	Faint trace
	2 b	1 0 1/00	—	—	" "
	3 b	2 0 1/00	G	G	Very marked
	4 b	4 0 1/00	G	G	" "
	5 b	None	G	G	Distinct
	6 bg	5 1/5	—	—	0
p-chlor-m-kresol	1 c	5 1/5	—	—	Trace
	2 c	1 0 1/00	G	G	Marked
	3 c	2 0 1/00	G	G	Very marked
	4 c	4 0 1/00	G	G	" "
	5 c	None	G	G	Trace "
	6 cg	5 1/5	—	—	0
Hexabrom-dioxy-diphenyl-carbinol	1 d	5 1/5	—	—	0
	2 d	4 0 1/0	—	—	0
	3 d	1 0 1/00	G	G	Marked
	4 d	2 0 1/00	G	G	Distinct
	6 dg	None	—	—	Trace
Sodium fluoride	1 e	2 %	G	—	Trace
	2 e	1 %	G	G	Marked
	3 e	.5 %	G	G	"
	4 e	.04 %	G	G	Faint trace
	5 e	None	G	G	0
	6 eg	2 %	—	—	0
Organisms autoclaved + tetrachlor-o-biphenol	1 fa	5 1/5	—	—	Faint trace
	2 fb	5 1/5	—	—	" "
p-chlor-m-kresol	3 fc	5 1/5	—	—	" "
Hexabrom-dioxy-diphenyl-carbinol	4 fd	5 1/5	—	—	" "
Sodium fluoride	5 fe	2 %	—	—	" "
NaCl sol. control	6 gg	None	—	—	0

while the last tubes give no reaction owing to complete reduction of the nitrite. From this it appears that no reduction occurs when the antiseptic is present in sufficient amount to kill the organism, while when the amount is less than is sufficient to kill the organism the reduction may yet be impeded, so that either no reduction occurs or the action stops at the formation of nitrite in place of proceeding to complete destruction of the latter. When the amount of antiseptic is very small it may have practically no effect, as e.g. in tube 4*e* of the above experiment where only a faint trace of nitrite is left when 0·04 % sodium fluoride was used. It may be remarked that while tube 1 of series *e* gave growth in the subculture made immediately before the addition of nitrite, this tube was sterile at the end of the experiment. This probably accounts for the non-reduction of nitrate, the organism having died off too soon after the nitrate was added. This series (*e*) shows all gradations from non-reduction of nitrate in the first tube (2 % NaF) to almost complete reduction of nitrite in the fourth (0·04 % NaF). That the absence of colour in the first tube is not due to complete reduction of nitrate to nitrite followed by destruction of nitrite was shown by adding nitrite ( $\frac{n}{20,000}$ ) to tubes of emulsion containing these amounts of the various antiseptics, and incubating at 37° C., when no reduction was found to occur.

*The action of organisms killed by the acetone process.*

The "Buchner-Albert-Rapp" acetone process was originally devised as a method of fixing yeast cells without destroying their power of fermenting sugar. In other words, its purpose was without destroying the fermenting enzyme itself to check the action of such proteolytic or other enzymes as might destroy the fermenting enzyme, and at the same time to destroy all power of proliferation. It was a modification of Albert's alcohol ether process which had been used for the same purpose but was subject to the disadvantage that alcohol interfered with the fermenting enzyme. In the Buchner process the yeast is washed, freed from water as far as possible by submitting it to a pressure of 50 to 100 atmospheres, and treated for about 15 minutes with acetone. It is then filtered free from acetone with the aid of pressure and suction, treated with a second quantity of acetone, freed from this and finally treated for a short time with ether. Having been freed as far as possible from ether, it is spread on filter paper and left exposed to the air for an

hour. It is then, to prevent it absorbing moisture from the air, placed in the incubator for 24 hours at 37° C. in order that the last traces of ether may evaporate. The final product is an almost white dry powder, which is found to contain only 5·5% to 8·5% of water. In adapting this method, the author was unable to make use of high pressure, but otherwise the method is the same. The bacilli were grown on 2½% agar in Kolle flasks and made into an emulsion with sterile water. About four times the bulk of acetone was then added and the bacilli immediately began to clump. They were allowed to settle and the supernatant fluid was then pipetted off, more added, and the mixture agitated for several minutes. The bacilli were again allowed to settle and the fluid changed. Finally ether was substituted for the acetone and after two washings the bacilli were dried. It is possible to shorten the process and obtain a larger yield by centrifugalising the mixture, to aid the precipitation of the organisms. Usually the emulsion was made in a large Jena glass test tube and transferred beads and all to a sterile 100 c.c. Jena flask for further treatment. The glass beads proved useful, not only in mixing the bacilli with the acetone but also in making them into an emulsion when they came to be used for experiments.

The product thus obtained at first formed a porous and friable cake, adherent to the bottom and sides of the flask, but on shaking the flask this cake was readily broken up by the glass beads to a fine yellowish grey powder. Its action on nitrites and nitrates was tested by making it into an emulsion with sterile water and incubating it with nitrate or nitrite solution. Equal amounts of emulsion and of sodium nitrate ( $\frac{n}{50}$ ) or sodium nitrite ( $\frac{n}{10,000}$ ) solution were placed in a sterile test tube. Similar amounts of emulsion and nitrate or nitrite solution were placed in separate tubes, and all the tubes were sealed. The emulsion and nitrite or nitrate solution which had been incubated separately were mixed immediately before testing and formed a control upon the tubes in which the emulsion had been incubated along with the salt. The following gives the result of such an experiment. The tubes were sealed in the flame, the lower end being immersed in cold water during the process and for some time after:

	Result when tested with naphthylamine acetate and sulphanilic acid
Emulsion and nitrate incubated together	Faint colour
Emulsion and nitrate incubated separately	Faint colour
Emulsion and nitrite incubated together	Marked colour
Emulsion and nitrite incubated separately	Marked colour

There is thus no difference between the solutions which were incubated along with the bacilli and the corresponding ones incubated separately. That is to say there has been no reduction of nitrate or nitrite by organisms killed with acetone.

#### SUMMARY AND CONCLUSIONS.

The organisms examined have been killed, (*a*) by temperatures which should not have destroyed the reducing enzyme, (*b*) by sodium fluoride and four other antiseptics, derivatives of phenol, and (*c*) by acetone. In each case there has been total destruction of the reducing power, any apparent production of nitrite from nitrate being too slight to justify the assumption that it is due to anything more than experimental error. Filtrates of fluid cultures have also failed to reduce. The results may be summed up as follows:

(1) The reduction of nitrite by certain dysentery bacilli is not due to a soluble extra-cellular enzyme.

(2) Dysentery bacilli and *B. coli* killed by a minimal degree of heat have little if any effect in reducing nitrites.

(3) Organisms killed by antiseptics or by acetone do not reduce nitrates and nitrites.

(4) Even where all the organisms are not dead, reduction may be prevented by antiseptics.

(5) The high antiseptic value of tetrachlor-o-biphenol, tetrabrom-o-kresol, p-chlor-m-kresol and hexabrom-dioxy-diphenyl-carbinol has been established for the organisms of the dysentery group.

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## THE ANTI-BACTERICIDAL ACTION OF THE BILE SALTS.

BY MAJOR S. LYLE CUMMINS, R.A.M.C.

BILE and the bile salts are substances of great importance in connection with typhoid fever. On the one hand, they are extensively used in differential media for the isolation of *B. typhosus* from the excreta, and in media designed to cultivate the bacillus from the blood; while on the other, the survival of the organism in the gall bladder and its association with gall stones and cholecystitis indicate that bile may play an important rôle in the etiology of the disease. It would appear therefore that a study of the mode of action of bile in culture media for the isolation of *B. typhosus* might, apart from its bearing on bacteriological technique, incidentally throw light on the far more important question of typhoid fever and the production of typhoid carriers.

The action of bile and the bile salts in favouring the growth of *B. typhosus* on differential media has been most ably investigated by Dunschmann<sup>1</sup>, but that observer did not extend his work to explaining the uses of bile in Blood-culture, beyond the fact of its "enriching" action on the growth of the typhoid bacillus, and its power of retarding the growth of certain other organisms.

Eppenstein and Korte<sup>2</sup> called attention to the anti-bactericidal action of bile, and Conradi<sup>3</sup>, recording further experiments to the same effect, pointed to this anti-bactericidal action as explaining the utility of bile in the culture of typhoid bacilli from the blood. A feature of Conradi's work was that he found bile to inhibit the bactericidal properties of serum, a point to be borne in mind, as Gildmeister, in a recent communication<sup>4</sup>, attributes the use of bile in typhoid blood-culture entirely to its haemolytic action, which he supposes to liberate anti-bactericidal substances from the disintegrated blood-cells.

<sup>1</sup> *Ann. Inst. Pasteur*, Jan. 1909, p. 29.

<sup>2</sup> *Münch. med. Wochenschr.* 1906, p. 1152.

<sup>3</sup> *Ibid.* p. 1361.

<sup>4</sup> *Arb. a. d. Kaiserl. Gesundheitsamte*, Feb. 1910.

Pies<sup>1</sup>, referring to Conradi's work above quoted, lays stress on the high concentration of nutrient matter in the serum as an important factor in the survival of typhoid bacilli.

Experiments carried out by the author in collaboration with Captain C. C. Cumming, R.A.M.C.<sup>2</sup>, led us to the opinion that, in the isolation of typhoid bacilli from the blood, the anti-bactericidal action of the bile salts was of far greater importance than any enriching quality that they might possess. The experiments about to be recorded were undertaken with a view to elucidating as far as possible this anti-bactericidal action, in the hope that some light might in this way be thrown on the survival of *B. typhosus* in "carriers," as well as on the properties of the bile salts as constituents of media for the culture of typhoid bacilli from the blood of cases.

**EXP. 1. Object.** To confirm previous work in demonstrating the anti-bactericidal action of sodium taurocholate.

An agar slope of B. T. "Rawlings" was emulsified in 10 c.c. of sterile normal salt solution.

A series of dilutions of this emulsion were prepared of the following strengths:—

1 in 1,000; 1 in 10,000; 1 in 100,000 and 1 in 1,000,000. It was desired to compare the bactericidal effect of a 1 in 8 dilution of normal blood in sterile water with that of the same strength of blood diluted with a 0·5% solution of sodium taurocholate. It was anticipated that on mixing known volumes of the blood preparations with equal volumes of successive dilutions of the typhoid emulsion, their relative bactericidal efficiency would be manifested in their power of sterilizing the bacterial dilutions in contact with them.

TABLE I.

		Dilution Series, Typhoid Emulsion			
		1 in 1,000	1 in 10,000	1 in 100,000	1 in 1,000,000
A.	Blood, 10 c.mm. Water, 60 c.mm. Dilution of emulsion, 10 c.mm.	{ Findings} on plates	+	-	-
B.	Blood, 10 c.mm. Taurocholate 0·5% solution, 60 c.mm. Dilution of emulsion, 10 c.mm.	{ Findings} on plates	+	+	+

+ = growth, - = sterile.

<sup>1</sup> *Arch. f. Hygiene*, 1907, LXII. p. 125.

<sup>2</sup> *Journ. Royal Army Med. Corps*, June 1910.

*Series A.* To 10 c.mm. of each bacterial dilution were added 10 c.mm. of freshly drawn blood and 60 c.mm. of sterile water.

*Series B.* To 10 c.mm. of each bacterial dilution were added 10 c.mm. of freshly drawn blood and 60 c.mm. of a 0·5% solution of sodium taurocholate. The four preparations from each series were incubated at 37° C. for 20 hours, and then spread on plates. The result is shown in Table I.

It will be seen that while the mixture of blood and water was able to sterilize a 1 in 10,000 dilution of typhoid emulsion, the presence of sodium taurocholate in a similar dilution of blood annulled all bactericidal effect on even so high a dilution of bacterial emulsion as 1 in 1,000,000.

This experiment has been frequently repeated and always with a like result. It might however be urged that the survival of the bacteria in contact with sodium taurocholate was due, not to any antibactericidal action of the salt but rather to the enriching power claimed for it by Dunschmann. To settle this point, it was decided to work out the enrichment power, if any, of the sample of sodium taurocholate under examination, leaving the action of blood out of the question.

EXP. 2. In separate test-tubes were placed 10 c.c. of ordinary peptone and salt solution and of a 0·5% solution of sodium taurocholate in peptone and salt. To each tube was added 10 c.mm. of an emulsion of B. T. "Rawlings," and both preparations were incubated at 37° C. for three days. A "count" of each preparation was then made, with the result that the bile salt peptone water tube contained 235,000,000 bacilli in 1 c.c. while the peptone water contained 249,000,000 per 1 c.c.

It is obvious, therefore, that the sample of sodium taurocholate under examination has no marked enriching effect in three days—in fact rather the reverse.

It may then be taken as proved that sodium taurocholate is able to inhibit the bactericidal action of normal blood.

Since it is well known that the bactericidal efficiency of the blood-fluids increases during the process of clotting and is greater in the serum than in fresh blood, it next became a question whether the bile salt acted by preventing the elaboration of bactericidal substances during clotting or interfered with their activities after elaboration.

EXP. 3. A broth-culture of B. T. "Rawlings" was diluted as in Exp. 1. A sample of blood was then withdrawn by finger-puncture, portion of it, in 1 in 4 dilution, treated at once, and the remainder allowed to clot and the serum treated in the same dilution after

10 minutes, 2 hours and 6 hours respectively. All preparations were incubated for 20 hours and plated.

The result is shown in Table II.

This experiment showed that sodium taurocholate acted, not by interfering with the formation of bactericidal substances, but by inhibiting their action.

Before proceeding further in the mechanism of this inhibition, it seemed important to ascertain whether this power was shared by the glycocholate of soda also, and whether the constituents of these salts, taurin, glycine, and cholalic acid, were able equally to interfere with bactericidal action.

TABLE II.

	Findings on plates	Dilution Series of Typhoid Broth Culture			
		1 in 1,000	1 in 10,000	1 in 100,000	1 in 1,000,000
A. Fresh blood, 5 c.mm. 0·5% taurocholate solution, 10 c.mm. Dilution of emulsion, 5 c.mm.	+	+	+	+	+
B. As above but with "10 minutes" serum	"	+	+	+	+
C. As above but with "2 hours" serum	"	+	+	+	+
D. As above but with "6 hours" serum	"	+	+	+	+

N.B.—The broth culture dilutions were kept at a temperature of 32° F. in the intervals of being used, to prevent multiplication of the bacilli.

EXP. 4. Dilutions of broth-culture of B. T. "Rawlings" were prepared as before.

Solutions containing 0·5% of each of the above substances were made in sterile water, and a mixture of one part of fresh blood in three parts of each solution was then prepared.

To 10 c.mm. of each blood-mixture was added 5 c.mm. of each dilution of the typhoid culture, and the preparations incubated and plated as before.

The result is shown in Table III.

It appears from the above that both sodium taurocholate and glycocholate possess anti-bactericidal qualities, while glycine, taurin and cholalic acid are without any such action, sterilization of the culture being active when blood is mixed with solutions of these substances.

The cholalic acid used was an old sample which had been long in the laboratory, and it will be desirable to further test this acid when a

TABLE III.

		Dilution Series of Typhoid Broth Culture			
		1 in 1,000	1 in 10,000	1 in 100,000	1 in 1,000,000
A.	Fresh blood 1 part } 10 c.mm. Sterile water 3 parts } Dilution of culture, 5 c.mm.	Findings } on plates }	+	-	-
B.	Fresh blood 1 part } 10 c.mm. 0·5% sol. of taurocho- } late of soda 3 parts } Dilution of culture, 5 c.mm.	"	+	+	+
C.	As above, but 0·5% taurin sol.	...	..	-	-
D.	As above, but 0·5% glycocholate of } soda sol.	..	+	+	+
E.	As above, but 0·5% glycine sol.	...	..	-	-
F.	As above, but 0·5% cholic acid sol.	..	+	-	-

reliable preparation is available. It is curious that, in view of the proved absence of anti-bactericidal action in taurin and glycine, and the activity in this respect of the taurocholate and glycocholate of soda, the cholic acid should be without this quality, but, assuming the sample used to be reliable, the above experiment certainly indicates that this is the case.

It is now time to return to the mechanism of this anti-bactericidal action of sodium taurocholate.

Regarding the disintegration of bacteria as a "complement-amboceptor" reaction, it may be assumed that bactericidal activity can be destroyed by preventing the action of amboceptor or of complement or both. The elucidation of this question is complicated by the difficulty of obtaining complement free from amboceptor, but this can to a certain extent be got over by comparing two sera of different bactericidal "titre."

EXP. 5. *Object.* To ascertain whether sodium taurocholate interferes with the sensitization of typhoid bacilli by amboceptor.

The serum of a rabbit possessing a considerable degree of immunity to *B. typhosus* and agglutinating it in a dilution of 1 in 200, was heated for 25 minutes at 60° C. to inactivate its complement. The serum of a normal rabbit was obtained at the same time and treated in the same way. A 24 hours' agar culture of B. T. "Rawlings" was emulsified in saline.

The following mixtures were then prepared :—

(1)	Heated immune serum	...	...	1 part
	0·5 % sodium taurocholate solution	...	...	1 part
	Typhoid emulsion	...	...	2 parts
(2)	Heated normal serum	...	...	1 part
	0·5 % sodium taurocholate solution	...	...	1 part
	Typhoid emulsion	...	...	2 parts
(3)	Normal salt solution	...	...	2 parts
	Typhoid emulsion	...	...	2 parts
(4)	Normal salt solution	...	...	1 part
	0·5 % sodium taurocholate solution	...	...	1 part
	Typhoid emulsion	...	...	2 parts

These mixtures contained, in all cases, the same concentration of typhoid emulsion, and where it was present, of sodium taurocholate. They were left in contact, over night, at room temperature to ensure sensitization of the bacteria, if this indeed could take place in the presence of the taurocholate, in the serum preparations.

The mixtures were then centrifuged for 45 minutes, the deposits washed in saline, and again centrifuged. After a final washing the deposits were emulsified in saline, the emulsions being "matched" by opacity to eliminate as far as possible fallacies arising through unequal multiplication and unequal "deposit"—the latter especially, as there was agglutination in *both* serum preparations, though of course this was more marked in the immune serum.

It was expected that if the bile salt had not interfered with the amboceptor in the heated immune serum, the bacteria in "mixture 1" would be so far sensitized as to be able to deflect complement to a greater extent than those placed in contact with normal serum. The saline emulsion was introduced as a "control" and the "bile salt—saline" mixture to make sure that the sodium taurocholate itself exerted no "sensitizing" action on the bacteria. The four emulsions were handed to Major W. L. Harrison, R.A.M.C., who very kindly undertook this part of the work. He examined them without knowing which emulsion was supposed to be the sensitized one, until the "key" was consulted after the experiment, and he reported as follows :—

"Complete deviation occurred when emulsion No. 1 was placed in contact with 1 in 50 complement, partial when No. 2 was ditto, and none when Nos. 3 and 4 were placed under the same circumstances."

"From your key, it appears that bile salt did not affect the amboceptor. Possibly there was enough amboceptor in your normal serum, in the quantity used, to sensitize your bacteria; hence the partial result with No. 2."

The fact that there was agglutination with the "normal" serum would support Major Harrison's surmise as to the possibility of the presence of amboceptors.

The experiment goes far to prove that amboceptor is not interfered with by sodium taurocholate, and the anti-bactericidal action of this salt is therefore probably exerted through an inhibition of complement.

EXP. 6. *Object.* To ascertain whether sodium taurocholate interferes with the action of complement.

The serum of the immunized rabbit used in Exp. 5 was heated for 20 minutes at 60° C. to destroy its complement.

Equal parts of this heated serum and an emulsion of B. T. "Rawlings" in saline were left in contact for 1 hr. at 37° C. The sensitized bacilli were centrifuged, the deposit washed in saline, and the resulting emulsion diluted in series from 1 in 10 to 1 in 1 million.

A mixture of normal human blood 1 part and 0.5% sodium taurocholate solution two parts, was then prepared and allowed to stand for 1½ hrs. 10 c.mm. of this preparation was added to an equal volume of each dilution of the emulsion of sensitized cells. At the same time, as a control, 10 c.mm. of normal salt solution was mixed with an equal volume of each bacterial dilution.

Both series were then incubated for 20 hrs. and plated.

It was anticipated that, if complement were still active in the blood-bile salt mixture, this would enable the already sensitized bacilli to be dissolved, and the higher dilutions of bacterial emulsion would be sterilized.

On plating, however, it was found that there was complete growth in all the dilutions up to 1 in 1 million, proving that the blood, when mixed with sodium taurocholate, was unable to "complement" the sensitized bacilli.

It is evident then that the anti-bactericidal action of sodium taurocholate depends on interference with the complement, and not on inhibition of the action of the amboceptor.

It is not suggested that the taurocholate can prevent the complementing and digestion of sensitized typhoid bacilli when the latter have been ingested by phagocytes. Such observations as have been carried out indicate that phagocytosis and intracellular digestion of typhoid

bacilli can both take place in contact with a 0·5% solution of sodium taurocholate in citrated normal salt solution, though the destructive action of the bile salt upon the blood elements renders the observation difficult and unsatisfactory. But short of interfering with phagocytosis, the "anti-complement" action of the bile salts may perhaps have an important rôle in typhoid fever and the production of "carriers."

It may be permissible to consider for a moment the conditions obtaining in—say—the third week of an attack of typhoid fever. The agglutinating power of the blood is now high and the clumped bacteria have been, to a great extent, filtered out of the general circulation. It is tempting to imagine them "held up" in considerable aggregations in the internal organs, such as the spleen, the liver and the adenoid tissue of the intestinal mucosa.

Probably the anchoring of "clumps" in these organs makes the work of phagocytosis both by leucocytes and tissue cells an easier task in some respects, but the ingestion of many virulent bacteria must also lead to the breaking down of leucocytes and, in all probability, the liberation of complement. In other words, at this time, there is probably an appreciable amount of extracellular solution of the typhoid bacilli; a surmise which is supported by the onset of the toxic symptoms characteristic of the later stages of the disease.

But while the liberation of complement leads, in most situations, to the extracellular solution of the already sensitized bacilli, the presence of bile at any given point would presumably prevent this solution, and enable even sensitized typhoid bacilli to survive and multiply. It is just in the positions where such an anti-bactericidal action of the bile is possible that foci of infection are found in typhoid carriers, e.g. throughout the hepatic area and in the mucosa and walls of the gall-bladder.

The hypothesis put forward, while perhaps too speculative to be of value in itself, may give point to the experiments here recorded, and emphasize the importance of further work on the possible rôle of the bile salts in typhoid fever and its sequelae.

ON THE VARIETIES OF *BACILLUS COLI* ASSOCIATED  
WITH THE HOUSE-FLY (*MUSCA DOMESTICA*).

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THE importance of flies in relation to the transmission of infectious diseases has been manifested in numerous instances; the incrimination of the house-fly, however, is as yet based upon by no means irrefutable grounds. Many observations have been made which must arouse grave suspicion, but the evidence hitherto adduced is far from conclusive. That house-flies can, and do convey infective material has been demonstrated both experimentally and under natural conditions, but the full significance of the house-fly as a disease-transmitter and the circumstances under which it assumes this rôle are still vague and indefinite.

The relation, in particular, of the house-fly to bacteria, pathogenic and otherwise, is not so thoroughly understood as might be desirable. It is well known that it may harbour a considerable variety of bacteria in its alimentary canal and carry an equal variety on the surface of its body, yet it is quite uncertain what organisms, if any, are particularly associated with the fly. It is not to be doubted that the bacterial flora of the fly's intestine may change under certain circumstances; at one time a particular group of organisms may be abundant and be replaced by others at some later period. The mutual relationship of these bacteria and their attitude towards organisms introduced into the intestine, are factors which must be considered in dealing with the problem of the house-fly as a carrier of pathogenic bacteria.

Only a very limited number of bacteriological examinations of flies under natural conditions has hitherto been made, and there has been no attempt to furnish a detailed account of such bacteria as have been

found. One of the earliest investigators to consider the relation of bacteria to flies was G. Marpmann (1884), who examined 230 flies. His method was to press a drop of fluid from the proboscis and from the anus, and to examine these in stained preparations. He determined the presence of bacteria in every one of the flies, but beyond this he did not go. In 1903 W. H. Horrocks examined a number of flies in which he found lactose-fermenting bacilli which he considered to be *B. coli communis*, as they did not ferment saccharose. Graham-Smith (1909) made a series of examinations of flies for the presence of colon bacilli and classified the bacilli so found according to MacConkey's four groups. He found that out of 94 house-flies 21% carried such bacilli, but only 4% (out of 56) contained them in their intestine.

The present investigation, which is only preliminary in nature, follows the lines adopted by Graham-Smith, but the characters of the organisms isolated have been studied in greater detail in accordance with the methods proposed by MacConkey (1909). Many of the flies examined were obtained from houses in which epidemic diarrhoea had occurred<sup>1</sup>, but various other sources were also drawn upon.

It has been shown very clearly by Graham-Smith (1910) that although many bacteria may adhere to the external surface of flies, they do not, in the case of non-sporing forms, survive there for more than a few hours. On the other hand, bacteria which have been ingested by the fly may remain in its intestine and be passed in its faeces for periods reckoned in days. The intestine must therefore be regarded as a more important vehicle of transit than the legs or surface of the body. In his experiments Graham-Smith found that, by feeding flies on pure cultures, *B. typhosus*, *B. enteritidis* (Gaertner) and *Vibrio cholerae* could be recovered from the intestine 6, 7, and 2 days later, respectively. Ficker (1903), Hamilton (1903) and Faichnie (1909a) have isolated typhoid bacilli from the intestine of flies caught in the vicinity of patients suffering from enteric fever. Faichnie (1909b), moreover, was able to recover typhoid bacilli from a fly which had become infected 16 days previously. These experiments and observations show that the typhoid bacillus may remain in the fly's intestine for a considerable period even under natural conditions.

Graham-Smith came to the conclusion that typhoid bacilli do not grow or multiply within the fly's intestine, and that they are more or less rapidly eliminated or destroyed. My own experiments in this

<sup>1</sup> I have to thank Dr G. Quin Lennane, M.O.H. Battersea, for affording me the opportunity.

respect lead to the same conclusion. That other bacteria, however, amongst which some pathogenic to man, may grow and multiply inside the fly, is not without the range of possibility. During the course of my experiments, I have found that certain varieties seem to be able to establish themselves in the fly's intestine, to the exclusion, sometimes, of other forms, and to remain there for a considerable time. Amongst these may be mentioned Morgan's *Bacillus No. 1* and *Bacillus paratyphosus*  $\beta$ . The former I have met with not infrequently and a few flies have yielded almost a pure culture of this organism. *B. paratyphosus*  $\beta$  I have found in two flies, which were being used for experimental purposes. They formed part of a batch of flies which were fed on a sample of faeces from a typhoid carrier. These two particular flies were examined 4 and 7 days respectively after they had fed on the faeces. *B. paratyphosus*  $\beta$  was recovered from the external surface and intestine of the first fly and from the intestine of the second, in each case in almost pure culture. The identity of the bacillus was confirmed by Dr F. A. Bainbridge. Bacteriological examination of the faeces on which the flies had fed failed to reveal the presence of *B. paratyphosus*  $\beta$ . Three subsequent examinations, as well as many previous examinations of faeces from the same patient gave negative results, so the conclusion cannot be avoided that the flies had become infected previous to the experiment. The flies had been kept under sterile conditions for a week before experiment. The source of infection could not be ascertained but it is apparent that the second fly must have carried the bacillus for at least 14 days, or that it had become infected from the first fly, which had carried it for at least 11 days. The point of importance is that at the end of that period the bacilli were still present in large numbers in the intestine. This, so far as I am aware, is the only record of this bacillus occurring naturally in flies. Morgan's bacillus No. 1 was obtained from flies by Morgan and Ledingham (1909) and appears to be a not uncommon inhabitant of the fly's intestine.

The numerous observations which have already been made on the subject clearly establish the fact that the house-fly may carry an enormous number and a great variety of bacteria and may inoculate those into materials on which it feeds. They have also shown that many of the bacteria pathogenic to man and animals may be carried in this way. It remains to determine the extent to which this means of transmission may occur in nature. To this end one of the most essential points appears to be a knowledge of the natural relation of flies to bacteria. A knowledge of the bacteria naturally occurring in flies,

their relation to each other and their relation towards other, possibly pathogenic, bacteria, which may from time to time be introduced, can hardly fail to be of importance in dealing with the subject of fly-borne disease. It is evident that, before any general conclusions can be drawn from experimental work with flies in their relation to the transmission of pathogenic bacteria, or from chance observations on the natural occurrence of such bacteria in flies, the above mentioned points will require some consideration.

With regard to the natural intestinal flora, it is certain that all the bacteria which may be isolated from a fly under ordinary circumstances cannot be regarded as constituting its natural flora. Feeding as it does continuously or intermittently on contaminated material, its intestine contains a large number of bacteria derived from its food. It is more than likely that a considerable proportion of these are simply passing through and never become resident. This becomes apparent when flies are fed for a length of time on sterile food. The characteristically faecal bacteria, such as the colon bacilli, tend to disappear under such circumstances and to be replaced by other organisms, frequently non-lactose-fermenting. The common occurrence of such organisms towards the end of experiments has given rise to the suspicion that they perhaps represent to some extent the natural intestinal flora of the fly. This suspicion is strengthened by the observation of Graham-Smith (1911) that non-lactose bacteria commonly occur in recently emerged flies. Dr Ledingham has also had a similar experience in a series of experiments, the results of which are in course of publication<sup>1</sup>. I am indebted to him for his information on this matter.

The majority of the flies examined in the present investigation were obtained from the dwelling rooms of houses in London. Several were also examined from the various outhouses in connection with the laboratory. The procedure was almost invariably the same. The flies were first well washed in sterile broth, then in 2% lysol or absolute alcohol for 10-20 minutes. They were then thoroughly washed in sterile water, dried over a flame and the whole alimentary canal was placed in broth. After incubation at 37° C. overnight the broth cultures were plated on MacConkey's bile salt medium, and from each plate about a dozen colonies were picked off and their characters determined. Altogether 145 specimens of *Musca domestica* were examined. 25 of these were examined individually, the rest in 23 lots of 5 to 7 each. Of the 96 plates which were made from these, 55 showed

<sup>1</sup> *Vide Journ. of Hyg.* xi. No. 3, 1911.

the presence of lactose-fermenting bacilli. 21 external cultures and 20 internal were negative. In 12 cases lactose-fermenting bacilli were found neither inside nor outside. Less than 75% of the flies may therefore be regarded as carrying colon bacilli either in their intestine or on the surface of their body. In 3 cases no growth of any kind was obtained from the surface or the intestine. In 9 cases there was a growth from the surface but not from the intestine, and in 5 cases a growth from the intestine but not from the surface. At least 5% therefore of the flies carried no aerobic bacteria capable of growing on MacConkey's medium.

Excluding the numerous cases in which two or more colonies of the same organism were picked off the same plate, colon bacilli were met with 77 times. These represented 27 different varieties and most of them possessed characters corresponding with those of some organism in MacConkey's list. Seven varieties not agreeing with any in that list were met with, but without exception only in isolated cases.

The chief difficulty encountered in classifying these organisms was with regard to the liquefaction of gelatine. The great majority have shown no trace of liquefaction at the end of a year. Some, however, have shown the first signs after six months. Only a few produced marked liquefaction within four months. The latter proved to be *Bacillus cloacae*, or Nos. 36 or 102 in MacConkey's list.

The indole test was performed twice in every case and found to be constant. The Voges and Proskauer's test was repeated in several doubtful cases. The motility was always investigated in a six hours' broth culture. The sugar fermentation reactions were found to be remarkably uniform. With only one exception, acidity and gas production were apparent within 48 hours in the case of glucose, mannite, saccharose and dulcite, and in the negative cases, no further change was noticed at the end of six days. The exception was a strain of *B. vesiculosus* which fermented dulcite after four days. With regard to lactose, fermentation was almost invariably slower, but only in two cases was it delayed beyond the fourth day. The same applies to adonite. With regard to inulin the result was negative even at the end of ten days except in one case, in which fermentation occurred within four days.

The following table shows the characters and numbers of the organisms investigated. They were all Gram-negative, gave acid and gas in lactose, glucose and mannite and produced acid and clot in litmus milk. They are numbered and named according to MacConkey's list.

TABLE.

		Numbers obtained									
		Gelatine	Mobility	Indole	Succharose	Dulcite	Adonite	Inulin	Voges and Proskauer	External	Internal
<b>GROUP I.</b>											
1. ...	...	-	+	+	-	-	+	-	-	1	1
2. <i>B. acidi lactici</i>	...	-	-	+	-	-	-	-	-	0	1
4. <i>B. Grünthal</i>	...	-	+	+	-	-	-	-	-	3	2
5. <i>B. vesiculosus</i>	...	-	-	+	-	-	-	-	-	2	0
7. ...	...	-	+	-	-	-	-	-	-	2	2
8. <i>B. coli mutabilis</i>	...	-	-	-	-	-	-	-	-	2	2
		Total								10	8
		Number of varieties								5	6
<b>GROUP II.</b>											
34. <i>B. coli communis</i>	...	-	+	+	-	+	-	-	-	5	10
35. <i>B. Schafferi</i>	...	-	-	+	-	+	-	-	-	1	1
36. ...	...	+	+	-	-	+	-	-	-	1	0
33a. ...	...	+	+	+	-	+	+	-	-	0	1
36a. ...	...	-	+	-	-	+	-	-	-	1	1
		Total								8	13
		Number of varieties								4	5
<b>GROUP III.</b>											
65. <i>B. oxytoccus perniciosus</i>	...	+	-	+	+	+	+	+	+	0	1
66. ...	...	-	-	+	+	+	+	-	-	0	1
67. ...	...	-	-	-	+	+	+	-	+	1	2
71. ...	...	-	+	+	+	+	+	-	-	6	7
72. <i>B. Neapolitanus</i>	...	-	-	+	+	+	+	-	-	2	0
74. ...	...	-	+	-	+	+	+	-	-	0	1
75. ...	...	-	-	-	+	+	-	-	+	1	1
66a. ...	...	-	+	+	+	+	+	-	-	0	1
		Total								10	14
		Number of varieties								4	8
<b>GROUP IV.</b>											
101. ...	...	-	-	+	+	-	+	-	-	0	1
102. ...	...	-	+	+	-	+	-	+	-	2	4
104. <i>B. gasoformans non-liquefaciens</i>	-	-	-	+	-	+	-	-	-	2	2
106. ...	...	-	+	+	+	+	-	-	-	1	2
108. <i>B. cloacae</i>	...	-	+	-	+	-	-	-	+	0	3
109. ...	...	-	+	-	+	-	-	-	-	1	1
106a. ...	...	-	+	+	+	-	-	-	-	0	1
108a. ...	...	-	+	+	+	-	-	-	+	1	0
		Total								7	14
		Number of varieties								5	8
		Total in all the groups								35	49
		Total number of varieties								18	23
											77
											27

Comparing the above table with that given by MacConkey (1909) it is apparent that a considerable similarity in respect of the colon bacilli exists between the bacterial flora of flies, and the bacteria met with in the faeces of man and other animals. The most striking feature is the marked preponderance of the characteristic faecal organisms *B. coli communis* and MacConkey's bacillus No. 71.

From the varied sources from which his organisms were derived, MacConkey found that those belonging to group III were greatly in excess of those belonging to the other groups and constituted 45% of the whole. Those of group IV were only about a third as frequent. From purely faecal sources the disproportion between these two groups is still more marked. In a general way, therefore, it may be considered that the bacilli of group III are more characteristically faecal organisms than those of group IV. The latter were obtained for the greater part from non-faecal sources. The varieties in groups I and II were more frequent in faeces than in other materials. Again in human faeces groups I, II and III were all about equally common, while group IV was comparatively rare. An examination of my table will show that each of the groups is about equally represented. There is not the comparative rarity of group IV characteristic of excremental material nor the rarity of groups I and II characteristic of other sources. This would indicate that the colon bacilli found in flies comprise a mixture derived both from faecal and other sources. Such a conclusion is in accord with preconceived notions.

With regard to the individual varieties, those most frequently met with by MacConkey were No. 71, No. 34 (*B. coli communis*) and No. 5 (*B. vesiculosus*), and these were the three organisms which occurred most frequently in excremental matter. They were also most frequent in human excrement. From other sources, however, the most common varieties were Nos. 71, 73, and 108 (*B. cloacae*). *B. coli communis* and *B. vesiculosus* were much rarer. Now in *Musca domestica* the most common varieties were 34 and 71 followed by 102, 4 and 7. The great frequency of *B. coli communis* can hardly be regarded as other than direct evidence of faecal contamination. The same may be said of No. 4, which was never found by MacConkey apart from faeces.

It is impossible to tell to what extent the flies in the present investigation have acquired contamination from human excrement. The number of bacterial organisms which have from time to time been isolated from human faeces and which appear capable of living for a longer or shorter period in the human alimentary canal is very great.

The same holds good in the case of several animals, and from the very incomplete evidence at present in existence, the intestinal floras of various mammals have very much in common. Differences and distinctive features have however not passed unnoticed, and with fuller knowledge the presence of a certain few bacteria might be sufficient to warrant the diagnosis of excremental matter from a particular animal or class of animals. This would demand more intimate acquaintance with the bionomics of bacteria, and in particular of those which are parasitic in man and animals. Modern methods have shown that bacteria under artificial conditions have very varied physiological requirements, and there is no reason to suppose that their requirements under natural conditions are any less varied. If such is the case, it follows that for each class or group of bacteria and for individual species or varieties of that group, there will be some environment which is an optimum and in which that particular species thrives and develops to the fullest extent. There can be no question that a great variety of physiological environment does exist in the alimentary canal of animals. Some organisms are known to require an extremely specialised environment, while others can accommodate themselves to a wide variety. For this reason the distribution of some organisms is extremely wide, while that of others is restricted. From biological considerations it may be assumed that each variety of environment will call forth the existence of a corresponding variety of organism and that the latter is conditioned by the former. There should therefore be just as many species or varieties of organisms as there are variations in environment. The different environments obtaining in the intestine of man and other animals should be sufficient to differentiate distinctive groups of bacteria and these groups would probably be readily isolated in the faeces were it not for the fact that a large number of bacteria probably simply pass through the intestine with the food, not actually growing and multiplying, but at the same time not being killed off in the process. It ought however to be possible by suitable experiment to elucidate this matter and I hope to be able to do so to some extent in the case of flies.

#### SUMMARY.

1. A study of the natural bacterial flora of the house-fly appears to be essential in forming a correct estimate of the part played by flies in transmitting pathogenic bacteria.
2. The house-fly may carry at least 27 varieties of *Bacillus coli*, by

far the most frequent of which are *B. coli communis* and MacConkey's bacillus No. 71.

3. As far as can be judged from the character of these colon bacilli the house-fly derives its bacterial flora equally from excremental matter and from other sources.

4. Certain non-lactose fermenting bacilli appear to be capable of multiplying in the intestine of the house-fly. Of these Morgan's bacillus No. 1 is a not infrequent inhabitant of the fly's intestine and *B. paratyphosus*  $\beta$  has been found on two occasions.

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SOME OBSERVATIONS ON THE ANATOMY AND FUNCTION  
OF THE ORAL SUCKER OF THE BLOW-FLY (*CALLI-  
PHORA ERYTHROCEPHALA*).

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(With Plates IV—VIII.)

IN the course of a long series of experiments on the distribution of bacteria by non-biting flies (*Musca domestica* and *C. erythrocephala*)<sup>1</sup> it became evident that such flies are able to filter off and reject the larger particles contained in the fluids on which they feed. This fact seems to have escaped the notice of most observers, and although the proboscis of the blow-fly has been a favourite subject of study for many years no observations appear to have been recorded which throw any light on the means by which the filtration is effected. In order to ascertain the mechanism by which the filtration is accomplished a large number of dissections of the proboscis of the blow-fly have been made, and experiments have been carried out on the living fly to test the degree of its efficiency.

The present paper deals with (A) the anatomy of the distal end of the proboscis, which is the part alone concerned in filtration, and (B) feeding experiments affording experimental evidence of the efficacy of the filter.

(A) *The anatomy of the distal end of the proboscis of the blow-fly.*

The proboscis of the blow-fly has been carefully described by Lowne (1895) and others, and consequently there is no necessity to describe in detail the principal parts of the structure<sup>2</sup>.

<sup>1</sup> Graham-Smith (1910-1911).

<sup>2</sup> Wherever possible the names applied by Lowne (1892-5) have been used.

Briefly the proboscis of the blow-fly consists of two parts, a proximal conical portion, the rostrum, and a distal half, the proboscis proper, or haustellum, which bears the oral sucker. The relationship of the structures, which compose the main portions of this organ, may be seen by reference to Pl. IV, fig. 1, which represents a schematic longitudinal section through the proboscis, constructed from drawings made from numerous dissections and serial sections which were studied in order to ascertain whether any valvular structures exist in the proboscis. These observations failed to reveal any valve-like structures.

The filtering mechanism is situated in the oral sucker or suctorial disc which is described by Lowne (1895, p. 136) as "a fleshy oval disc, deeply cleft at its anterior margin. The edges of the cleft are continuous with the margins of the groove in the theca, and are united as far as the edge of the disc by a remarkable bead and channel joint. The thick edge of one lobe, or labellum, of the disc fits into a corresponding cylindrical channel in the other. The distal or oral surface of the disc is channelled by the well-known pseudo-tracheae. In the centre is a deep longitudinal fissure, which extends into the tubular mouth situated between the labrum and the theca. The proximal or aboral surface of the sucker is convex and covered by setae; those near its margin are very long and form a fringe."

Lowne (p. 390) describes the mouth as "a cylindrical tube extending from the thecal (or discal) sclerites to the prepharyngeal tube, which may be regarded as its posterior limit or isthmus faucium." The deep cleft between the two lateral halves of the oral disc into which the mouth opens he terms the prestomum (p. 143).

The dissections and feeding experiments described in this paper show that the liquid food is sucked into the pseudo-tracheae and drawn through the collecting channels and along the gutters of the prestomum into the mouth, and it seems probable that crop contents and saliva may be forced at will in the reverse direction for distribution over solid food which has to be moistened and dissolved. In order to explain the process of sucking food into the mouth the structures involved, namely the oral surface of the suctorial disc, the pseudo-tracheae, and the prestomal cleft, must be described in detail.

The pseudo-tracheae, varying in number between 28 and 32, run transversely across the labellum or lobe of the oral sucker. They form three sets. The seven anterior pseudo-tracheae run into a common longitudinal collecting channel which opens into the prestomum between the first and second prestomal teeth, and the posterior eight

to twelve in the same way run into a common posterior collecting channel, which opens into the prestomum at its shallow posterior extremity. The central pseudo-tracheae terminate in short channels which run directly into the prestomum without the intervention of common collecting channels. By this arrangement all the pseudo-tracheae are made to converge to the prestomum. The arrangement of the pseudo-tracheae is clearly shown in Pl. VII, fig. 9, which is a photograph of the oral surface of the expanded suctorial disc of a blow-fly with 30 pairs of pseudo-tracheae. On each side the anterior eight run into a common anterior collecting channel, and the posterior twelve into a common posterior collecting channel, while the ten central pseudo-tracheae are continued separately into the prestomum. Fig. 5 shows ten pseudo-tracheae running into the posterior common collecting channel.

From the points at which they cease to be tubular in structure the collecting channels are continued along the prestomal cavity to the mouth as grooves or gutters, whose lateral walls are formed by the prestomal teeth.

#### *The pseudo-tracheae.*

The pseudo-tracheae are deep furrows or incomplete membranous tubes embedded, more or less deeply according to the degree of its inflation, in the substance of the oral surface of the labellum, but under any conditions projecting sufficiently to produce distinct ridges. Along the apex of the ridge the wall of the pseudo-trachea is lacking so that the interior of the tube is in communication with the oral surface of the disc through a very narrow zigzag fissure. The lumen of the tube is kept open by means of incomplete chitinous rings running transversely round the tube, each of which has one fork-like bifid extremity, enclosing a rounded space between the prongs, and one extremity slightly expanded and flattened so as to resemble the tail of a fish. The rings are arranged in such a manner that along each side of the central fissure the bifid extremity of one ring alternates with the expanded extremity of the next ring. In consequence of this arrangement, which is very clearly seen in preparations treated with potash for the purpose of demonstrating the chitinous structures, the margin of the pseudo-trachea at each side of the central fissure has a deeply indented or scalloped appearance. The really effective entrances into the pseudo-tracheae are through the spaces between the bifid extremities of the rings and not through the narrow continuous zigzag

fissure, which is at any time extremely narrow and is probably closed during the act of feeding, as will be explained later.

The pseudo-tracheae gradually diminish in diameter as they approach the margins of the disc, and the size of the forked extremities of the rings and consequently of the spaces between them also diminishes though not to a corresponding degree.

The term "interbifid space" is used to indicate the area enclosed between the forks of the bifid extremity of a ring.

Fig. 7 illustrates two consecutive rings with their bifid and flattened extremities, and Fig. 6 illustrates a side view of one of these rings. Fig. 13 is a photograph of several consecutive pseudo-tracheal rings which have been treated with potash and compressed. The terminations of the consecutive rings are well shown. Fig. 11 is a photograph of the oral surface of the disc of a blow-fly after treatment with potash showing portions of four pseudo-tracheae. The longitudinal fissures of the pseudo-tracheae and the forked extremities of the rings and interbifid spaces can be clearly seen. Fig. 12 is a photograph of a section of the disc showing four pseudo-tracheae cut transversely. The chitinous rings, the openings of the longitudinal fissures and the ridges caused by the projection of the tubes above the surface are clearly shown.

The pseudo-tracheae of several of the common non-biting flies closely resemble each other, though they exhibit slight and apparently unimportant differences in their structure. The average measurements of the various parts in six common species are as follows.

	Interbifid spaces			
	Pseudo-tracheae		Diameter near the proximal ends of the pseudo-tracheae	Diameter near the distal ends of the pseudo-tracheae
	Diameter at proximal end	Diameter at distal end		
<i>Calliphora erythrocephala</i>	...	.02	.01	.006 .004 mm.
<i>Sarcophaga carnaria</i>	...	.02	.01	.005 .004 mm.
<i>Lucilius caesar</i>	...	.02	.01	.006 .004 mm.
<i>Fannia (Homalomyia) canicularis</i>	.016		.008	.006 .004 mm.
<i>Ophyra anthrax</i>	...	.016	.008	.006 .004 mm.
<i>Musca domestica</i>	...	.016	.008	.004 .003 mm.

The cuticle lining the oral surface of the labellum dips down into the pseudo-tracheae through the longitudinal fissure and also forms the lining of these tubes, as may be seen by reference to Fig. 12. In passing downwards into a pseudo-trachea the cuticle accurately follows its chitinous margins, being closely adherent not only to the chitinous sides of the interbifid spaces but also to the intervening elevations

between them produced by the projection of the expanded ends of the alternate rings, and the application to them of the adjacent forks of the neighbouring rings on either side.

Owing to this arrangement a remarkable series of folds is produced in the cuticle forming channels or grooves leading into the interbifid spaces. If the cuticle is traced along the edge of the longitudinal fissure from the bottom of one interbifid space to the bottom of the next it can be observed to be very closely attached to the chitin along the base of an interbifid space and up the side of a fork to its pointed extremity. It then passes over the expanded portion of the alternate ring and down the adjacent fork of the next ring, binding the two forks mentioned and the expanded portion of the intermediate ring into an elevated mass which lies between the deep depressions of the interbifid spaces. The arrangement described can be most easily understood by reference to Fig. 15, a longitudinal section through a pseudo-trachea just to one side of the central fissure. The depressions caused by the cuticle adhering to the bases of the interbifid spaces are continued outwards as folds or grooves in the cuticle for a considerable distance which are gradually lost on the surface of the labellum. Each "interbifid groove" thus forms a well defined channel leading into the pseudo-trachea through the interbifid space with its long axis at right angles to the line of the pseudo-trachea. The deepest part of the groove is at its entrance into the pseudo-trachea, and at this point it loses its groove-like character and becomes a tunnel, though still communicating with the surface by a very narrow slit. When the proboscis is erected by slight pressure on the head and the oral sucker viewed with a microscope these grooves can be easily seen as regularly placed channels running at right angles to each pseudo-trachea.

Though difficult to describe the arrangement can be easily understood by reference to Fig. 4, representing a dissection of a portion of a pseudo-trachea. On the right-hand side the integument of the oral surface of the labellum has been removed so as to show a portion of the pseudo-trachea with the alternate bifid and flattened extremities of the chitinous rings and the membrane lining the interior of the tube stretching between them. On the left-hand lower portion the appearance of the surface integument is represented. Two interbifid grooves leading to their interbifid spaces are shown. Between the interbifid spaces are elevated masses, each of which is produced by a fold of the integument enclosing the flattened end of a ring and the extremities of the adjacent forks on each side. In the left-hand upper portion of the diagram is

shown the appearance of these structures as seen by transmitted light so as to indicate the relationship of the integument to the rings.

Fig. 19 is a photograph of a wax model of short segments of two pseudo-tracheae. The cuticle and internal lining membrane have been stripped off the outer side of the pseudo-trachea on the left exposing the chitinous rings. On the inner side of this pseudo-trachea and over the whole of the pseudo-trachea on the right the cuticle is represented *in situ*. The interbifid grooves leading into the interbifid spaces can be easily seen. In Fig. 12 illustrating transverse sections of four pseudo-tracheae the interbifid groove is indicated on the left side of each pseudo-trachea, but is perhaps best seen in the central ones. In each case the point of bifurcation of the chitinous ring is indicated by a dark spot above which the forks are curved inwards. From the point of bifurcation a distinct line, which represents the reflection of the cuticle at the base of the interbifid groove, passes obliquely upwards and outwards to the surface of the integument. Fig. 6 illustrates diagrammatically the condition seen in transverse sections. Fig. 16 is a photograph of the oral surface of part of a disc including portions of two pseudo-tracheae. The interbifid grooves can be seen as somewhat oval-shaped regularly arranged areas on each side of the pseudo-tracheae. The zigzag line in the centre is the median longitudinal pseudo-tracheal fissure.

Anthony (1874), Wright (1884) and Lowne (1895, p. 395) all regarded the interbifid grooves as suckers. The latter figured them as blind sacs attached to the forks of the rings with openings into the pseudo-tracheae only. None of these authors seemed to regard them as channels leading into the pseudo-tracheae.

The fact that these interbifid grooves are really channels leading into the pseudo-tracheae can be demonstrated however by a very simple experiment. If the proboscis of a blow-fly is placed in alcohol, formalin or other preserving agent, and the suctorial disc is later mounted in water under a cover-glass and examined with the aid of a microscope the grooves can be clearly seen. As the specimen begins to dry air bubbles often form in the slight depressions on the oral surface of the disc between the pseudo-tracheae. As the drying continues the bubbles run into the interbifid grooves and through them into the pseudo-tracheae, clearly showing that the grooves lead into the pseudo-tracheae.

*The collecting channels.*

It has already been stated that the anterior and posterior sets of pseudo-tracheae run into common collecting channels, and that the central pseudo-tracheae also run into separate closed channels. These channels, which are kept open by incomplete chitinous rings without bifid extremities, communicate with the exterior by narrow fissures which are continuations of the longitudinal fissures of the pseudo-tracheae. Since there are no interbifid spaces there are no interbifid grooves or other openings into these channels. Each channel opens into its corresponding gutter between the prestomal teeth in a remarkable manner, the more deeply situated portions of the proximal rings being expanded and prolonged towards the prestomum, so as to form a spout-like opening to the channel.

Fig. 5 illustrates the posterior common collecting channel of one labellum with ten pseudo-tracheae opening into it. It will be noticed that throughout the greater part of its length the extremities of the rings are either quite plane, or slightly expanded, or possess only the rudiments of forks. Consequently the channel opens to the exterior by a longitudinal fissure only. At its proximal end the chitinous bars representing the rings are elongated and form a shallow groove leading towards the discal sclerite which forms the side of the entrance of the mouth. Two pseudo-tracheae opening through their own collecting channels into gutters between the prestomal teeth are shown at the right-hand side. In Fig. 2 the proximal portions of three of the central pseudo-tracheae with their collecting channels terminating in spout-like openings are illustrated, and Fig. 18 is a photograph showing several of these channels and gutters.

When at rest the oral surfaces of the labellae or oral lobes are in apposition, but during feeding they are spread out over the surface of the food so as to form an oval disc. In order to attain this position that part of the oral surface of the labellum adjoining the prestomum, which is situated just external to the teeth, in fact all that area bordering the longitudinal sulcus, is capable of being bent through a right angle. It is over these highly flexible regions of the suctorial disc, which are invariably bent during the act of feeding, that the pseudo-tracheae are converted into closed collecting channels.

*The prestomal teeth.*

On each side of the prestomum is arranged a series of rows of chitinous teeth, usually ten in number (Fig. 18). The central rows each consist of three teeth. The innermost teeth are the strongest and are articulated at their proximal extremities on to the chitinous side of the lateral plate of the discal sclerite, while their distal free extremities are bifid. Except at their bases they are free from investment with integument. The intermediate teeth are longer than the inner and their distal extremities are placed directly external to those of the inner set. Their distal extremities are bifid. Immediately behind the free extremities of the inner set these teeth branch, and the two branches pass behind and to the sides of the inner set to be inserted into the discal sclerite. The upper thirds only of these teeth are free from integument. The outer teeth resemble the intermediate set in their general shape and disposition, but are longer. Their distal extremities are bifid, but their proximal branched extremities are not inserted into the discal sclerite, but seem to articulate with it indirectly through the intervention of plates of chitin. Only the distal ends of the outer set are free from integument.

The arrangement of these teeth will be best understood by reference to Fig. 2 which represents the teeth as seen from the oral aspect, and Fig. 3 which represents them as seen from the aboral aspect after the removal of the overlying structures, and indicates the extent to which they are free from integument.

The spaces between the rows of teeth form the gutters, leading into the prestomum, which have already been mentioned. The gutter is bordered by the teeth of the inner set, whilst its floor is formed by the branches of the intermediate and outer sets of teeth, and the integument investing these structures.

The arrangement described is only found near the centre of the prestomum. On either side of the two or three central pseudo-tracheae each tooth of the outer set is represented by two bars of chitin, which are not united at their distal extremities (see Fig. 2). Still further from the centre the outer set is lacking, while at either end of the series both the outer and intermediate sets are lacking.

The arrangement of the teeth varies greatly in different species. *C. erythrocephala* has as described three teeth in each row, *S. carnaria* has four, and *L. caesar* has three. In *M. domestica*, *F. canicularis* and

*O. anthrax* there seems to be only one definite series corresponding to the inner set of *C. erythrocephala*. In these species the sets which are lacking seem to be represented by modified plates of chitin.

When fluid food is being taken the teeth merely aid the conveyance of the fluid into the mouth by assisting in the formation of the gutters. They may be used however under suitable conditions in scraping the surfaces of hard substances to render their solution more easy. In order to bring the teeth into action as scrapers the lobes of the suctorial disc have to be more widely separated than they usually are when liquid food is being taken. When the teeth are in action as scrapers the prestomal cavity is open to the surface, and if sucking efforts are made probably large particles can pass into the mouth.

#### (B) *Feeding Experiments.*

If hungry flies are fed on drops of syrup or other fluids they rapidly suck up large quantities. The general behaviour of flies during the act of feeding and subsequently has been previously described (Graham-Smith, 1910, pp. 5-11; 1911, p. 41) and need not be repeated in detail. In all cases the suctorial disc is inflated and the lobes spread out so that the oral surfaces of the labellae are nearly in one plane. If viewed from its oral surface the disc presents the appearance seen in Fig. 9, the adjacent sides of the lobes being pressed together so that the prestomal cavity is almost completely closed.

If the flies are fed on shallow drying drops of somewhat concentrated syrup containing finely ground Indian ink deposited on glass, proboscis marks, recognizable as white areas where the ink deposit has been removed, can frequently be observed (Fig. 20). These areas correspond with the shape of the inflated proboscis showing that the margins, and probably the greater part, of the suctorial disc are closely applied. The firmer the application of the disc to the surface supporting the food the more completely are the walls of the prestomal cavity pressed against one another. Hence under these circumstances no material can enter directly into the mouth but has to be conveyed into it through the agency of the pseudo-tracheae and collecting channels.

If the head of a blow-fly is removed and the proboscis erected by slight pressure on the head and fixed in that condition with plasticine it is possible to obtain an excellent view of the expanded disc. In this position each lobe of the disc is convex in its transverse diameter and the entrance to the prestomal cavity is recognizable as a longitudinal

sulcus slightly expanded near its centre. By applying a cover-glass to the oral surface of the disc it can be readily shown how pressure exerted on the disc closes the prestomal cavity in proportion to the degree of the pressure.

Under natural conditions flies probably seldom have the opportunity of feeding on large drops but suck up thin films of moisture and consequently feed with their proboscides so closely applied that the longitudinal prestomal sulcus as well as the longitudinal fissures of the pseudo-tracheae are to a great extent obliterated. Under these conditions it seems impossible that food should enter the mouth except through the interbifid grooves, and that this is actually the case can be proved by experiments with suitable fluids. If flies are allowed to suck at films of partially dried Indian ink they often remove from the glass only those portions which lie immediately under the interbifid grooves. In such cases beautiful patterns like gratings are left on the glass. Fig. 21 is a photograph of a portion of one of these patterns. The fly has applied the proboscis firmly to the surface so that the outline of the disc is clearly visible. It may also be seen that the longitudinal prestomal sulcus was almost completely closed. The lines of the pseudo-tracheae are marked by double parallel rows of regularly placed clear oral areas separated by thin black lines. Each of these areas from which the pigment has been removed by suction represents the space covered by an interbifid groove. Fig. 22 is a photograph of a portion of a similar pattern more highly magnified. It will be noticed that no traces of the zigzag fissures running longitudinally along the pseudo-tracheae can be seen. These fissures are entirely obliterated by the pressure of the proboscis on the surface causing the free ends of the rings to meet, as can be readily understood by reference to Fig. 12.

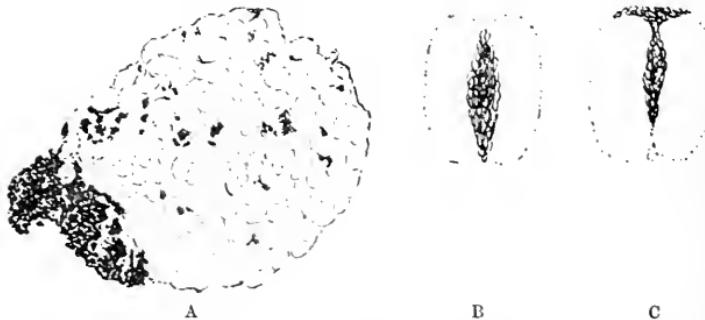
The longitudinal axis of each pseudo-trachea is marked by a zigzag black line. On each side of this line are clear areas caused by the removal of pigment through the interbifid grooves. The way in which this pattern is produced is best understood by reference to Fig. 4 (left-hand side).

The broader black lines represent the inter-pseudo-tracheal plain areas of the disc.

If fed on a drop of moderate depth the proboscis does not seem to be so closely applied to the surface on which the drop is placed, though the disc is in an erected condition.

If a fly is allowed to feed on a large drop containing particles of various sizes it often sucks up all the fluid and leaves the larger particles

in an irregular mass at one edge of the drop. When the area originally covered by the drop is examined with the aid of a lens it is found to be covered with numerous clear oval proboscis marks, indicated by fine lines of pigment at their peripheries. It is evident therefore that the fly at each application of its proboscis has sucked up and swallowed the fluid and smaller particles which are capable of passing through the interbifid spaces, and that the suction has caused the larger particles to adhere to the disc. After all the fluid has been swallowed the larger particles adhering to the proboscis are deposited either through the cessation of the suction, or by a small quantity of fluid being forced in a reverse direction to wash off the deposit.



- A. The appearance of a drop of syrup, containing in suspension particles of Indian ink of various sizes, after being sucked by a blow-fly. All the larger particles have been left in an irregular mass at the edge where the fly stopped feeding. Numerous oval proboscis marks cover the rest of the surface, and here and there a few of the larger particles are left stranded. (Drawn from a photograph.)
- B. Lens-shaped mass of pollen grains, a cast of the longitudinal sulcus, deposited by a fly while sucking a drop of fluid containing very large numbers of pollen grains in suspension.
- C. T-shaped mass deposited under the same conditions.

Occasionally when large particles such as pollen grains are present in great numbers in shallow drops of fluid the fly deposits the larger particles at frequent intervals. Such deposits may be irregularly rounded masses or may sometimes be lens- or T-shaped. The latter condition probably indicates that the disc has not been applied very closely, for under such conditions the longitudinal prestomal sulcus is slightly open and may be recognized in inflated specimens as a groove slightly expanded near its centre. The stem of the T which is often slightly expanded near its centre is produced by particles which

have been drawn into the longitudinal sulcus and the cross piece by particles which have been drawn against the walls of the anterior fissure of the disc. Often the cross piece of the T is lacking, when the majority of the larger particles have been drawn into the longitudinal sulcus, and a lens-shaped deposit results.

The mode of formation of these deposits can be followed when flies are fed on drops of fluid containing very large particles, for the currents set up can be easily examined. Since suction is created in all the pseudo-tracheae crossing the oral surface of the disc the particles are drawn towards the disc from all points, and if too large to enter the interfibid grooves form a ring round the circumference of the disc. Those which are drawn towards the anterior extremity are sucked into the anterior fissure. If the proboscis is very closely applied they remain at its outer margin, but if it is less closely applied they may be drawn into the partly closed longitudinal prestomal sulcus and form a cast of that cavity. Consequently either the outline of the proboscis is marked by stranded particles or the longitudinal sulcus is also indicated. In these cases the fly has probably ceased sucking when it lifts its proboscis. On the other hand the irregular masses of deposit, which have been described, are formed by the particles running together as the fly lifts its proboscis while still sucking.

The fly is apparently capable of slightly lifting the outer edge of the disc while feeding by means of the epifurca. Occasionally in this way particles may pass under the centre of the outer margin of the disc.

A large number of experiments were carried out in order to ascertain the size of the largest bodies which could be swallowed. For this purpose flies were made to feed on drops of various fluids containing in suspension bodies of definite size and shape such as spores of moulds, pollen, etc. Immediately after feeding they were killed and dissected and the crop and intestinal contents examined for the presence of the suspended particles. It was found that the spores of *Nosema apis* and of various moulds measuring up to 0.006 mm., in fact all bodies measuring in their smallest axis less than the diameter of the interfibid space, could be readily swallowed.

The case is however different with larger bodies such as pollen grains. Many feeding experiments were carried out with emulsions of the contents of bees' colons, containing many easily recognizable bodies of various sizes, including pollen grains in various stages of digestion. In most cases pollen grains, except of very small size, could not be detected in the crop or intestinal contents of the flies. On rare occasions

however numerous pollen grains were found, both in the crop and in the intestine. On closer examination many of these were found to be empty flattened shells, readily distorted to a slight degree, but afterwards regaining their shape. Such bodies could be easily sucked through the interbifid spaces. Still more rarely one or two apparently undigested pollen grains were found. Also in some experiments with recently gathered pollen from the pollen baskets of the healthy bees the grains ( $.02 \times .04$  mm.) could be detected in the crop or intestinal contents of a small proportion of the experimental flies. No object measuring more than  $.02$  mm. in its smallest diameter was ever swallowed.

It was found that objects of comparatively large size were more frequently ingested when suspended in viscid fluids, such as honey, or when flies were endeavouring to extract the fluid from semi-solid masses composed of large particles.

In experiments on the part played by flies (*M. domestica*) in the dispersal of parasitic eggs Nicoll (1911, p. 18) found that the flies were "apparently unable to ingest particles of larger size than  $.045$  mm." His tables show that the ova of *Hymenolepis diminuta* ( $.07 \times .065$  mm.—33 experiments), *Toxascaris limbata* ( $.08 \times .07$ —12 experiments) and *Ankylostoma caninum* ( $.06 \times .04$  mm.—8 experiments) were never swallowed. Occasionally the ova of *Trichuris trichiurus* ( $.05 \times .025$ —1 out of 12 experiments) and of *Taenia marginata* ( $.035 \times .035$ —4 out of 19 experiments) were ingested. The ova of *Taenia serrata* ( $.035 \times .035$ ) however were sometimes ingested in considerable numbers (13 out of 31 experiments). Judging from the writer's experiments it is undoubtedly rare for particles whose smallest measurements exceed the diameter of the larger interbifid spaces to be ingested even in small numbers. Occasionally however such particles may be swallowed in considerable numbers. Two explanations are possible, either that the particles are forcibly drawn into the pseudo-tracheae or that they pass directly into the mouth. By reference to Figs. 4, 11, 16 and 19 it will be noticed that the interbifid spaces and grooves are not placed symmetrically on opposite sides of the pseudo-tracheae, but a narrow diagonal fissure, a branch from the main longitudinal fissure of the pseudo-trachea, connects the openings of each pair placed nearly opposite to one another. Though under normal conditions the food passes through the interbifid spaces and bodies larger than these spaces are filtered out, yet by strong suction and a slight degree of distortion the fissures connecting two

nearly opposite interbifid grooves may be forced open and allow of a body equal in diameter to the length of a line connecting the two most distant points of the interbifid spaces to be drawn through. The length of this line is nearly equal to the transverse diameter (.02 mm.) of the pseudo-trachea. Consequently it is possible that in this way bodies which just fill the lumen of a pseudo-trachea may be drawn through the pseudo-trachea into the mouth. Further, if the pseudo-tracheal rings are capable of considerable distortion slightly larger bodies just capable of engaging two opposite interbifid grooves may be sucked in and along the pseudo-trachea, if it is distorted in such a manner that the longitudinal fissure is opened to allow of its passage.

According to the writer's experiments however bodies too large to pass along the pseudo-tracheae are very rarely ingested.

The presence of ova of tape-worms in the intestinal canal of *M. domestica* cannot be explained in this way. These objects are extremely attractive to flies, which may suck at segments of tape-worms for several hours in order to extract their contents (Nicoll, 1911, p. 20). The flies appear to make great efforts to swallow the ova and probably the prestomal cavity is at times open so that the ova pass directly into the mouth without passing through the pseudo-tracheae. This view is supported by the curiously uneven results obtained by Nicoll, who for example in one series of experiments fed seven flies on ruptured segments of *T. serrata* and found 400 ova in the intestines of two flies, two ova in one fly, and none in the other four flies. The most likely explanation seems to be that in the latter five flies the filter acted efficiently, whereas in the two former ova were allowed to pass into the mouth while the prestomal cavity was open. Possibly in their endeavours to swallow these ova the flies attempt to use their teeth to reduce their size. In order to do so the labellae have to be so widely separated that the prestomal cavity is open, consequently if suction is made during the process of scraping large particles may pass into the mouth.

All the observations hitherto made indicate that under most conditions the filter acts very efficiently and prevents the entrance of particles larger than .006 mm. in their smallest diameter into the mouth of the blow-fly. Exceptionally a few larger particles may be drawn forcibly through it, or pass directly through the prestomal cavity into the mouth.

*Summary.*

All the non-biting flies examined, *C. erythrocephala*, *M. domestica*, *S. carnaria*, *F. canicularis*, *L. caesar* and *O. anthrax*, possess a filtering apparatus situated in the pseudo-tracheae of the suctorial disc. The anatomy and action of this filter have been most thoroughly studied in *C. erythrocephala*. The suctorial disc is grooved by pseudo-tracheae which end near its centre in closed collecting channels. The latter open into furrows or gutters formed by the peculiar disposition of the prestomal teeth on the walls of the prestomal cavity. The opening of the mouth is situated at the base of the cavity. During natural feeding the lobes of the suctorial disc are pressed together so that the lumen of the prestomal cavity is obliterated, and no food can enter the mouth except through the collecting tubes. The pseudo-tracheae are channels kept open by chitinous rings situated in their walls. Each ring has one bifid extremity, enclosing between the horns the "interbifid space," which forms an opening of definite size into the pseudo-trachea. A fold in the cuticle, the "interbifid groove," leads to each interbifid space.

The fluid food is sucked first along the interbifid grooves through the chitin-lined interbifid spaces into the pseudo-tracheae. Particles of larger diameter than the interbifid spaces (.006 mm.) are usually prevented from entering the mouth and are rejected. The fluid and smaller particles are drawn along the pseudo-tracheae, through the collecting channels and gutters between the prestomal teeth into the mouth. By means of strong suction two opposite interbifid grooves may be made to communicate with each other owing to the lateral fissures connecting with the longitudinal pseudo-tracheal fissure being forced open, and consequently a few larger particles, up to .02 mm. in diameter, may be drawn into the pseudo-tracheae.

Certain relatively large and very attractive objects, such as the ova of tape-worms, too large to pass through the filter, may occasionally be swallowed. Such objects probably pass directly into the mouth, when the prestomal cavity is open, during the prolonged sucking efforts made by the flies.

The large number of experiments which have been made leave little room for doubt that under natural conditions, especially when the fly is feeding on a thin film of moisture, the filtering apparatus works with a high degree of efficiency.

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## DESCRIPTION OF PLATES IV-VIII.

Fig. 1. The right lateral half of the proboscis of the blow-fly divided in the middle line, and seen from the cut surface. The diagram has been reconstructed from dissections and serial sections. The mouth, prepharyngeal tube and pharynx are shaded.

1. Oesophagus. 2. Pharyngeal tube. 3. Salivary duct. 4. Fulcrum (with pharyngeal muscles). 5. Salivary valve. 6. Apodème of the labrum. 7. Hyoid sclerite. 8. Flange at proximal end of ligula. 9. Cavity of prelabrum (passing up to prepharyngeal tube). 10. Thyroid sclerite and contained muscles. 11. Paraphysis. 12. Ligula. 13. Hypoglossal sclerite. 14. Cavity of prelabrum. 15. Salivary gland of oral disc. 16. Prestomal teeth and prestomal cavity. 17. Labellum showing pseudo-tracheae, the anterior and posterior sets opening into common collecting channels. The epifurca can be seen running downwards behind the pseudo-tracheae. 18. Lateral plate of discal sclerite with nodulus (black). 19. Anterior portion of prelabrum with contained muscles.

The smaller figures are transverse sections at A-A and B-B, and are numbered as in the larger figure.

Fig. 2 represents four rows of prestomal teeth and the corresponding portion of the labellum seen from the oral surface. In the upper part of the figure three of the central pseudo-tracheae, showing the alternate bifid and flattened ends of their rings and the longitudinal fissures, are represented. Each passes into a collecting tube with non-bifid rings which terminates by a spout-like opening between the distal

extremities of two rows of prestomal teeth. The inner, shortest set of teeth are lightly shaded. They are unbranched and articulate with the lateral plate of the discal sclerite by their strong proximal extremities. The teeth of the intermediate set, which are more darkly shaded, branch behind the distal extremities of the inner set. The branches diverge to each side of the corresponding inner teeth to articulate with the lateral wall of the discal sclerite. The teeth of the outer set are most darkly shaded. The two central teeth of this set branch behind the distal extremities of the intermediate set. The branches diverge widely behind those of the intermediate set, but do not articulate directly with the discal sclerite. At each side of the figure this set is represented by separate chitinous bars which do not unite to form definite teeth.

The teeth of the inner set form the side walls of gutters whose floors are formed by the branches of the intermediate and outer sets of teeth and the integument covering them. Fluids drawn through the collecting tubes pass along the gutters into the mouth.

The tendinous chords described by Lowne (p. 395) are indicated between the pseudo-tracheae.

Fig. 3 represents a view of a single collecting tube and its corresponding teeth from behind, after the removal of the integument of the aboral surface of the disc, to show the relationship of the teeth to the cuticle. The distal halves of the inner set of teeth and the distal thirds of the intermediate set of teeth are free from integument.

Fig. 4 represents a dissection of a portion of a pseudo-trachea. On the right-hand side the integument of the oral surface of the labellum has been removed so as to show a portion of the pseudo-trachea with the alternate bifid and flattened extremities of the chitinous rings and the membrane lining the interior of the tube stretching between them. On the left-hand lower portion the appearance of the surface integument is represented. Two interbifid grooves leading to their interbifid spaces are shown. Between the spaces are elevated masses, each of which is produced by a fold of the integument enclosing the flattened end of a ring and the extremities of the adjacent forks on each side. In the left-hand upper portion of the diagram is shown the appearance of these structures as seen by transmitted light so as to indicate more clearly the relationship of the integument to the rings.

Fig. 5 illustrates the arrangement of the chitinous structures of the posterior common collecting channel of one labellum with 10 pseudo-tracheae opening into it. Throughout the greater part of the length of the common collecting channel the extremities of the rings are either quite plane or slightly expanded or possess only the rudiments of forks. At its proximal (right-hand) end the chitinous bars representing the rings are elongated and form a shallow groove leading towards the discal sclerite which forms the side wall of the entrance to the mouth. Two pseudo-tracheae opening through their own collecting channels into gutters between prestomal teeth are shown on the right-hand side.

Fig. 6 is a side view of a pseudo-tracheal ring. At its right-hand end the ring has a flattened expanded extremity; at its left-hand end a bifid extremity. The opening of the longitudinal fissure is seen between the flattened end of the ring and the tips of the forks. The arrangement of the fold of integument forming the interbifid groove is indicated by means of shading.

Fig. 7 represents two consecutive pseudo-tracheal rings, showing the relationship of their bifid and flattened extremities, as seen from the oral surface of the disc.

Fig. 8 is a schematic representation of a transverse section through the oral surface of

- part of labellum and prestomal cavity. The free extremities of the teeth are shown projecting above the integument (dotted line).
- Fig. 9 is a photograph ( $\times 66$ ) of the oral surface of the erected suctorial disc of a blow-fly. The pseudo-tracheae and anterior and posterior common collecting channels are well seen.
- Fig. 10 is a photograph of the oral lobe of the blow-fly treated with potash and flattened out. The pseudo-tracheae can be clearly seen as well as some of the prestomal teeth and the gutters between them.
- Fig. 11 is a photograph ( $\times 340$ ) of part of the oral surface of the labellum treated with potash showing portions of four pseudo-tracheae. The flattened and bifid extremities of alternate pseudo-tracheal rings are well seen.
- Fig. 12 is a photograph ( $\times 600$ ) of a transverse section of part of the oral surface of a labellum. Four complete pseudo-tracheae are included in the section. In each case the chitinous ring and the opening of the longitudinal fissure is very distinct. It happens that in each case the bifid extremity is situated on the left side of the pseudo-trachea. The point of bifurcation is indicated by a dark spot above which the forks are curved inwards. From the point of bifurcation a distinct line, which represents the reflection of the cuticle at the base of the interbifid groove, passes obliquely upwards and outwards to the surface of the integument (see Fig. 6).
- Fig. 13 is a photograph ( $\times 700$ ) of ten consecutive chitinous pseudo-tracheal rings treated with potash and compressed, showing their alternate flattened and bifid extremities.
- Fig. 14 is a photograph ( $\times 700$ ) of two separated and partially distorted pseudo-tracheal rings.
- Fig. 15 is a photograph ( $\times 600$ ) of a longitudinal section through a pseudo-trachea slightly to one side of the median line. The interbifid spaces and the manner in which the integument passes over and binds together the flattened extremities of the rings and the contiguous forks of the adjacent rings on each side can be clearly seen.
- Fig. 16 is a photograph ( $\times 1300$ ) of the oral surface of a part of a labellum including portions of two pseudo-tracheae. The interbifid grooves are seen as regularly placed oval markings on each side of the pseudo-trachea. The zigzag longitudinal fissure is also indicated (see Fig. 4).
- Fig. 17 is a photograph ( $\times 770$ ) showing a side view of three pseudo-tracheae (with bifid rings) passing into their collecting channels (with non-bifid rings) which run down between the teeth.
- Fig. 18 is a photograph ( $\times 226$ ) of part of the side of the prestomal cavity, showing rows of teeth and the gutters between them. The spout-like openings of the collecting tubes can be very distinctly seen.
- Fig. 19 is a photograph of a wax model of short segments of two pseudo-tracheae. The cuticle and internal lining membrane have been stripped off the outer side of the pseudo-trachea on the left exposing the chitinous rings. On the inner side of this pseudo-trachea and over the whole of the pseudo-trachea on the right the cuticle is represented *in situ*. The interbifid grooves leading into the interbifid spaces can be easily seen.
- Fig. 20 is a photograph of proboscis marks produced by a fly feeding on a thin layer of Indian ink spread on glass. The position of the anterior cleft is indicated in every proboscis mark. The marks show that in each case the proboscis has been firmly and evenly applied.
- Fig. 21 is a photograph ( $\times 77$ ) of a portion of a proboscis mark left by a fly attempting to suck up a layer of partially dried Indian ink deposited on glass. The outline of the suctorial disc is clearly shown. The marks indicating the position of the longitudinal

sulcus are very narrow, showing that the prestomal cavity was almost completely closed. The lines of the pseudo-tracheae are marked by double rows of regularly placed clear oval areas, separated by thin black lines. Each of these areas, from which the pigment has been removed by suction, represents the space covered by an interbifid groove.

Fig. 22 is a photograph of part of a proboscis mark similar to that shown in Fig. 21, more highly magnified ( $\times 770$ ). The longitudinal axis of each pseudo-trachea is marked by a zigzag black line, showing that the longitudinal fissure was closed. On each side of the zigzag black line are clear areas produced by the removal of the pigment through the interbifid grooves. Their shapes are very clearly defined. The way in which this pattern is produced can be readily comprehended by reference to Fig. 4 (left-hand side). The broad black lines, separating the clear areas, represent the inter-pseudo-tracheal plane areas of the disc.

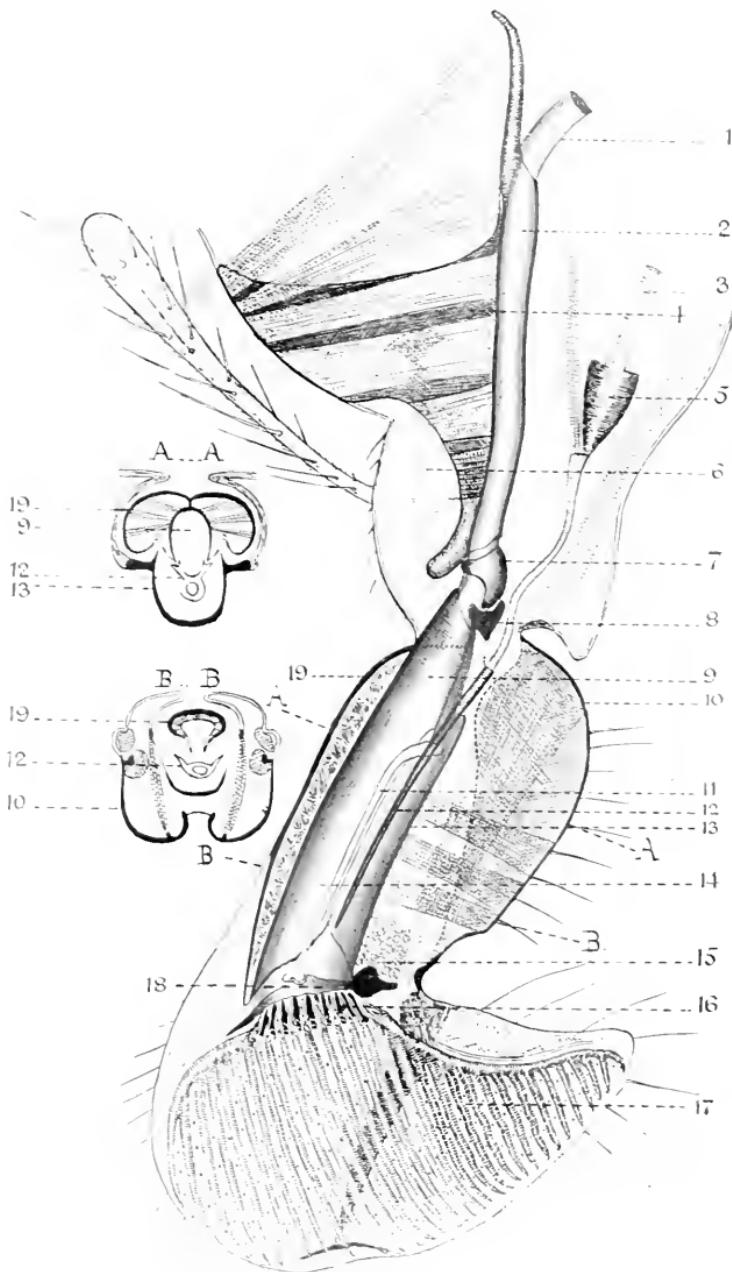


Fig. 1.



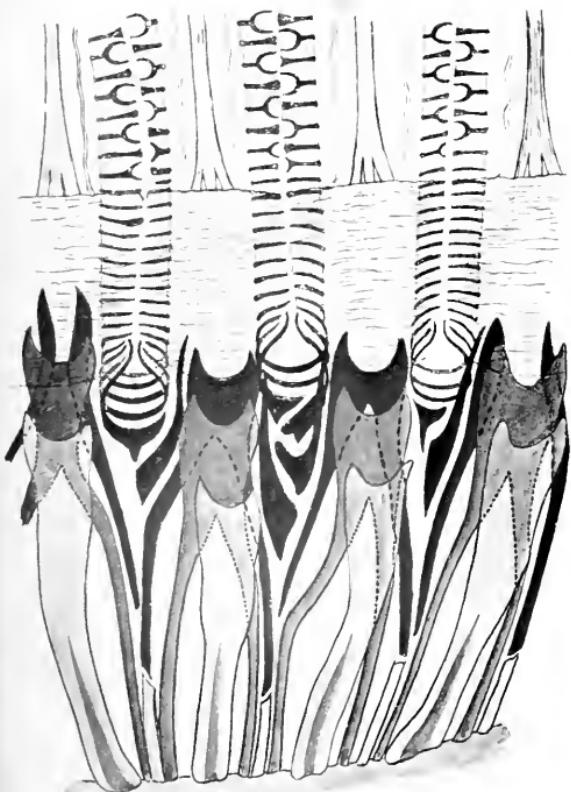


Fig. 2.

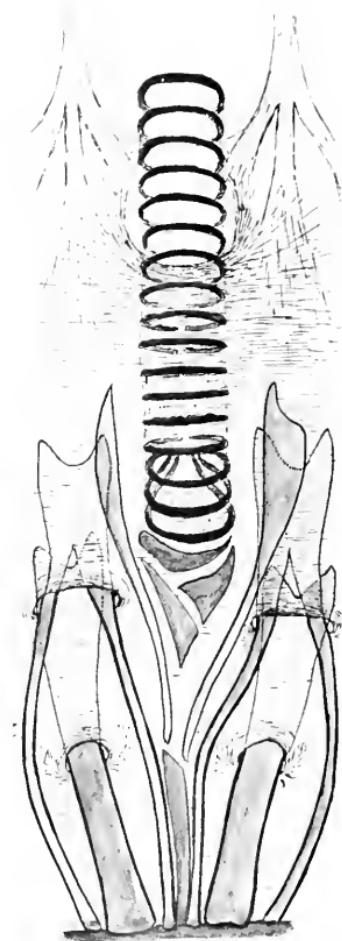


Fig. 3.



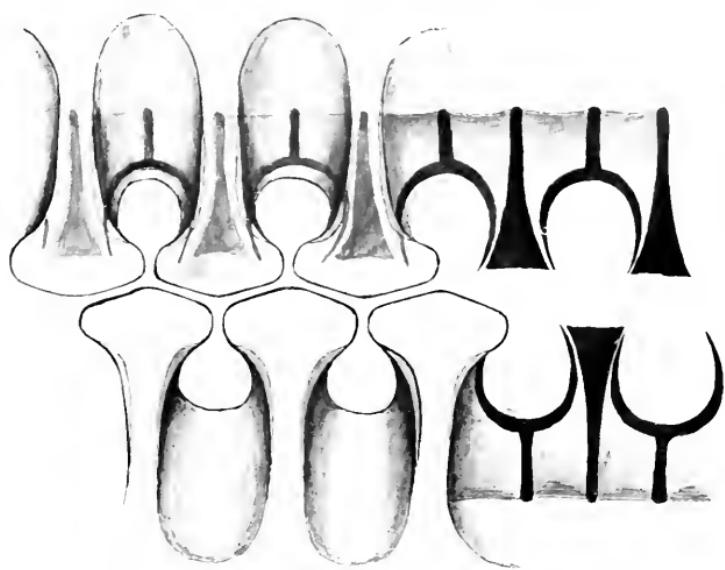


Fig. 4.

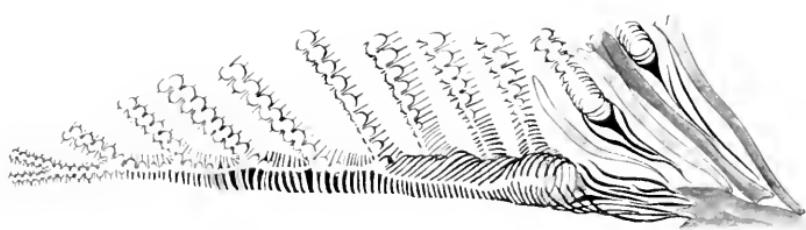


Fig. 5.



Fig. 6.



Fig. 7.



Fig. 8





Fig. 9.



Fig. 10.

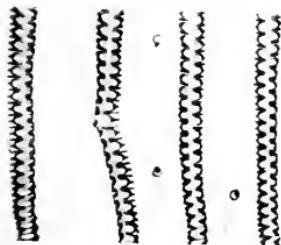


Fig. 11.



Fig. 12.



Fig. 13.

Fig. 14.



Fig. 15.



Fig. 16.

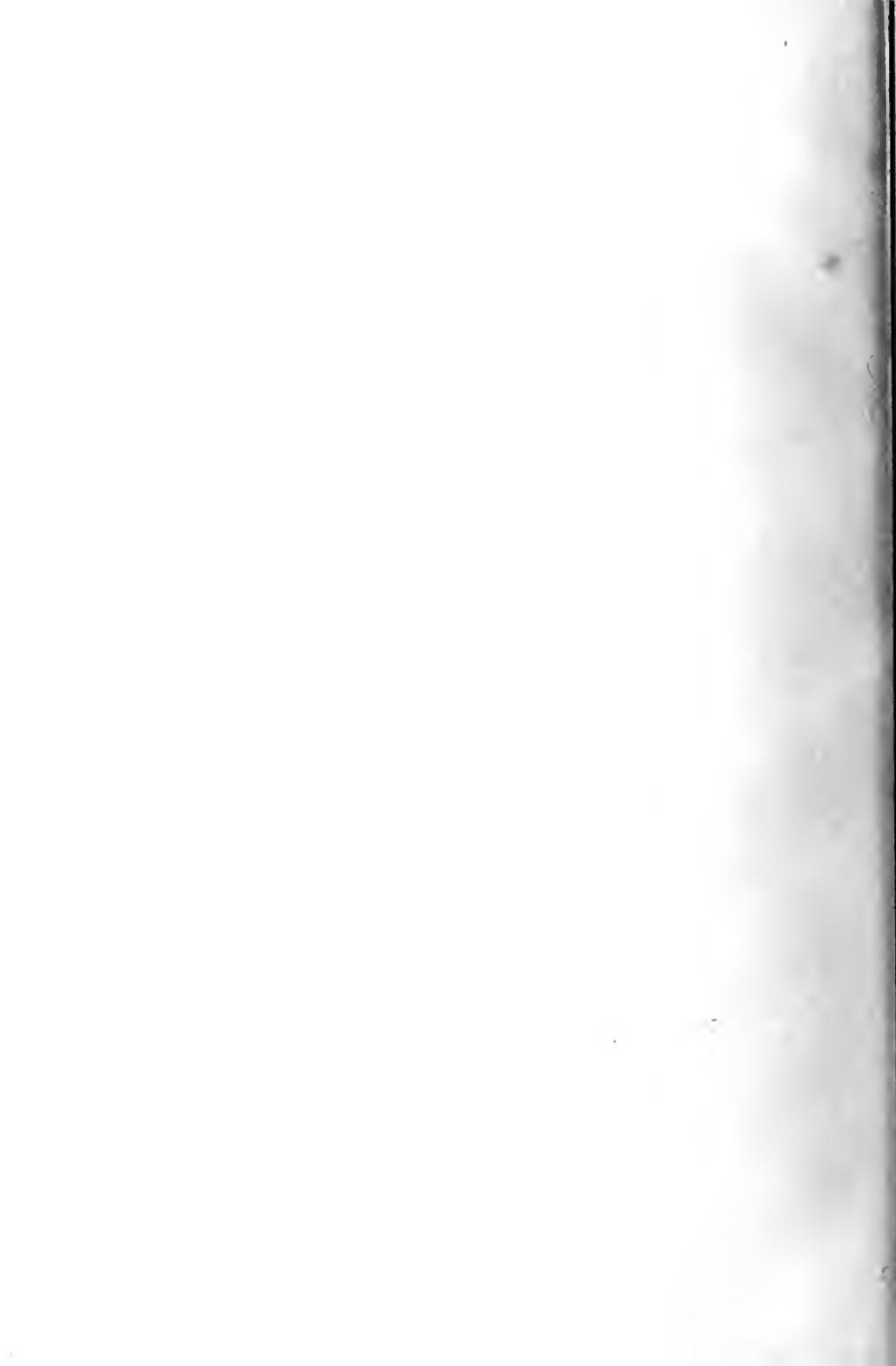




Fig. 17.

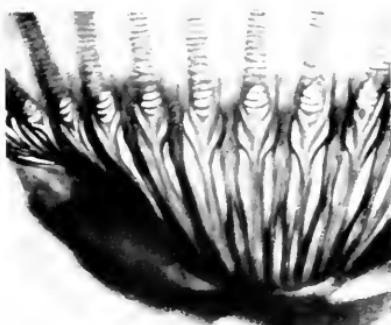


Fig. 18.



Fig. 19.



Fig. 20.

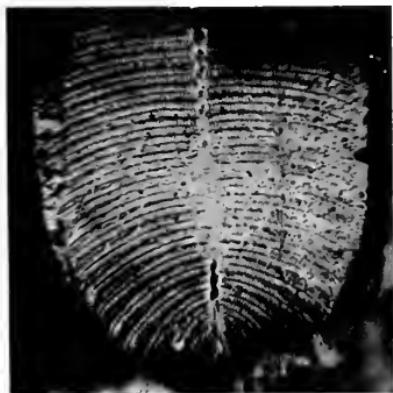


Fig. 21.

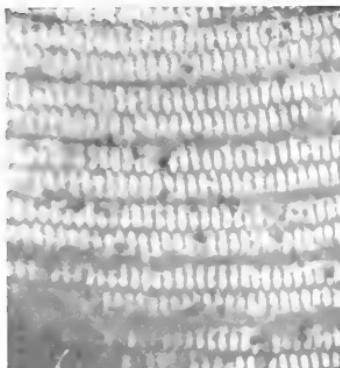


Fig. 22.



## VARIATIONS IN THE VIRULENCE OF DIFFERENT STRAINS OF *BACILLUS DIPHTHERIAE*.

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THE occurrence of non-virulent strains of bacilli, which are indistinguishable except by animal experiment from virulent diphtheria bacilli, makes it especially interesting to investigate the varying pathogenicity of this bacillus. Owing to their complete lack of pathogenicity for laboratory animals, the exact relationship of the non-virulent types of *B. diphtheriae* to the virulent forms of this organism, cannot yet be considered as settled, although Arkwright (1910) has succeeded in obtaining a slight but very definite development of antitoxin in the blood of a horse immunised with non-virulent strains.

In this paper no cognisance will be taken of the non-virulent forms. The experiments here recorded deal solely with the variations in virulence which are found to exist among strains which exhibit a definite pathogenicity for guinea-pigs.

The pathogenicity or virulence in a general sense has been examined by means of "whole" broth cultures containing both bacilli and the toxin formed by them, but an attempt has also been made to determine whether such differences in lethal power as occur between different strains are due chiefly to differences in the amount of toxin in the cultures, or to differences in the virulence proper of the bacillus, *i.e.* the activity of the bacillus after inoculation in the toxin-free state into the animal body.

### *Difference in the Pathogenicity of different strains.*

Many writers on this subject have recorded great variations in the pathogenicity of different strains, *e.g.* v. Behring (1901), Bardach (1895), Martin, L. (1898), Pennington (1907), and the matter is thoroughly

discussed by Graham-Smith and Dean in *The Bacteriology of Diphtheria*, (Nuttall and Graham-Smith (1908)).

Graham-Smith (1904) and Cobbett (1901), on the other hand, found a remarkable uniformity in the lethal dose of the strains which they isolated during two large epidemics of diphtheria at Cambridge. Cobbett out of 68 virulent strains, found only two which when recently isolated did not kill a guinea-pig of 250 grms. in two or three days, when 0·1 c.c. of a two-day broth culture was injected subcutaneously. (In four instances only, 0·5 c.c. was the smallest dose used.) The two strains of exceptionally low virulence were rapidly raised to the same degree of pathogenicity as the remainder, after one or more subcultures in broth.

Graham-Smith amongst 88 virulent strains isolated, found one which did not kill within two or three days with the smallest dose used. The doses injected varied from 0·1 to 0·3 c.c. but the majority of strains killed in a dose of not more than 0·2 c.c. With only one strain, 0·1 c.c. did not kill the experimental animal in 12 days, but the growth was very poor and subculture in broth did not materially increase its vigour.

It is to be noted, however, that these writers do not record any observations as to variations in virulence above these limits, *i.e.* when smaller doses than 0·1 c.c. were used. Moreover all the strains were isolated during two epidemics in the same town and their work shows the high degree of virulence of *B. diphtheriae* during these two epidemics.

Williams (1902) also noted the occurrence of strains of low virulence which attained a higher grade of virulence when the growth on broth had been improved by subculture.

Smith, Th. and Walker, E. (1896) tested the virulence of different strains by estimating the accumulation of toxin in broth cultures, and came to the conclusion that the virulence of different strains was very uniform. They did not, however, test the pathogenicity of cultures containing bacilli, and their conclusions are remarkable when one considers the enormous differences in the toxin-yield from broth cultures which have been obtained with different, or even the same, strains by many workers, *e.g.* Park (1896), Dean (1908), Madsen (1907).

I have not infrequently met with strains of low pathogenicity. In a school epidemic which I investigated (Arkwright, 1908), of 13 virulent strains which were isolated, only four were of such a degree of virulence that 0·1 c.c. of a two-day broth culture killed a guinea-pig of

250 grms. within four days and 2 c.c. in 48 hours. Three other strains when given in a dose of 0·1 c.c. killed the animal in ten days whereas a dose of 2 c.c. was fatal within four days. The remaining six strains were non-lethal when 0·1 c.c. was injected, but a dose of 2 c.c. was fatal within four and a half days.

At that time it seemed possible that this prevalence of strains of low virulence, might be causally connected with the large number of completely non-virulent strains found in the same epidemic (seven strains were non-virulent out of 20 strains which were isolated), and it seemed not unlikely that different degrees of virulence might prevail in different epidemics.

In the case of a small epidemic in a school from which I have recently examined material, five cases of diphtheria occurred. From these patients three strains of *B. diphtheriae* were isolated and tested for virulence. All were found to be of low virulence, when tested on several occasions. Although a large dose of 2 c.c. or 2·5 c.c. killed a guinea-pig rapidly, and a control animal to which antitoxin was given was unaffected, yet 0·1 c.c. proved to be a sublethal dose.

In another series of 37 virulent strains from various sources 25 were lethal when 0·1 c.c. and the remainder when 2·5 c.c. were injected.

#### *Method of estimating pathogenicity.*

In estimating the pathogenicity<sup>1</sup> of a strain of *B. diphtheriae* by the injection of "whole" two-day cultures (bacilli and toxin together) several difficulties were met with.

No doubt the exact composition of the broth in which the bacilli are grown makes a considerable difference in the results obtained by injecting whole cultures, and occasionally strains which are generally of low pathogenicity attain a much higher grade when grown on a different batch of broth, but when this happens there is sometimes reason to believe that the strains of high pathogenicity in cultures made at the same time, and in the same broth, are correspondingly exalted. Graham-Smith and Cobbett attach great importance to the use of sugar-free broth, but other workers have found that so long as the broth culture remains alkaline in reaction, the presence of a small amount of sugar does not hinder the formation of toxin (*e.g.* Smith, Th., Park and Williams).

<sup>1</sup> In this paper when referring to my own work the term "Pathogenicity" is used when "whole" broth cultures, "Toxicogenicity" when toxin free from bacilli, and "Virulence" when washed bacilli, have been employed for injection.

In this investigation the cultures in broth were found to remain alkaline. The broth employed was made from bullock's heart with 1% of Witte's peptone added, and rendered just alkaline to litmus paper. That the variations in the broth used were not an important cause of the differences in the results with the different strains, was shown by the fact that all degrees of virulence were found with the same brew of broth.

The period of incubation was two days at 37° C.

*Estimation of the amount of growth in broth.*

The growth in broth is obviously not always equally great when the same strain is used, and with different strains the amount and character of the growth varies very much.

Williams, Cobbett and Graham-Smith attribute the low virulence of their exceptional strains to the scanty growth in broth when they were recently isolated. In the case of my strains of low pathogenicity the growth was usually, but not always, small, and was not less than that of some strains possessing a higher lethal power. Training the bacilli to grow in peptone broth will sometimes increase the pathogenicity somewhat, but it is possible that other changes than increased ability to grow on artificial media may be induced in this way.

In order to estimate the amount of growth 10 c.c. of the bouillon cultures were centrifuged in a tube drawn out to a small calibre in its lower half and graduated in hundredths of a c.c. in this part. The amount of growth as shown by the deposit measured in this way was not proportionate to the virulence.

It was not found possible to count the bacilli in a broth culture on account of the clumping of most strains.

The following strains were used:

Strain No. I.	11 Bb	Isolated 24 Jan. 1911 from a diphtheria convalescent.
II.	66	," 2 Feb. 1911 from a school "carrier."
III.	134 X	," 21 Oct. 1909. Diagnosis swab.
IV.	134 Z	," 21 Oct. 1909. " "
V.	19 AT	," 25 Feb. 1911 from a diphtheria convalescent.
VI.	21 AN	," 25 Feb. 1911 " " "
VII.	R 50	," 6 Dec. 1910. Case of diphtheria. "
VIII.	R 4	," 7 Dec. 1910. " "
IX.	166	Skin infection by <i>B. diphtheriae</i> .
X.	27	Isolated 14 Mar. 1911. Diphtheria convalescent.
XI.	Dkth	," 12 Jan. 1911. " "
XII.	NE 30	," 16 Mar. 1911. " "
XIII.	R 70	," 31 Mar. 1911. Case of diphtheria.

The majority of the strains were chosen on account of their low virulence, but the extremes of virulence met with are represented. Some of the strains have been examined at considerable intervals of time with the object of ascertaining whether the degree of virulence remained fairly constant.

The results of injection of the "whole" two-day cultures are shown in Table I.

In this table the dose stated to be the M.L.D. is that of a two-day broth culture which caused death within four days. In another column is shown the sub-lethal dose, *i.e.* the highest dose which allowed the animal to survive. The approximate amount of deposit from 10 c.c. of the culture is also shown. The guinea-pigs were all between 240 and 250 grms. in weight. The doses used were usually 0·01, 0·02, 0·1, 0·5 and 2·5 c.c.

In Table II are recorded a few of the observations in which several strains of different degrees of virulence were tested on the same day in the same broth, showing that large variations in virulence were obtained with the same quality of broth.

In considering the results shown in Table I, it is seen that of the seven strains, I, II, IX, XI, V, VI, VII, each of which was examined on several occasions, four, viz. I, II, IX and V, remained fairly constant in pathogenicity. Of these I and II were of high pathogenicity, having a very small M.L.D. varying at most from 0·01 to 0·02 c.c. in the case of strain I, and from 0·02 to 0·1 c.c. in the case of strain II. The other two constant strains IX and V were of low pathogenicity, the M.L.D. varying from 0·5 to 2·5 c.c. Of the three remaining strains (XI, VI and VII) which were examined on more than two occasions, the M.L.D. varied from 2·5 to 0·1 c.c. Strain X was tested on only one occasion. Strain VIII was tested twice and on both occasions was of low pathogenicity.

The amount of deposit obtained by centrifuging 10 c.c. of culture varied for different strains between 0·0025 and 0·02 (1 to 8), but the amount of deposit was not proportionate to the pathogenicity of the culture, for the M.L.D. of culture for these strains varied from 2·5 to 0·01 (250 to 1). Thus strains I and II gave deposits only varying from 0·005 to 0·02 c.c. (1 to 4) and the M.L.D. for the same two strains varied from 0·1 to 0·01 c.c. (100 to 1).

In the case of strain IX the amount of deposit varied from 0·0025 to 0·005 c.c. with a M.L.D. never less than 0·5 (tested on four occasions). In the same way strain V gave a deposit of 0·0025 to 0·0075 c.c. but the M.L.D. was never less than 0·5 c.c.

TABLE I.

Strain and date of isolation	Dates of experiments	Deposit from 10 c.c. of culture	M.L.D. of "whole" culture	Day of death	Sublethal dose	Result of do.
No. I 24 Jan. 1911	4 Feb. 1911	—	0·1	Second	—	—
	11 Feb. ,,	—	0·02	Third	—	—
	16 Feb. ,,	0·015	0·01	Third	—	—
	15 May ,,	0·005	0·02	Second	—	—
No. II 2 Feb. 1909	9 Dec. 1909	—	0·1	—	—	—
	3 Feb. 1911	—	0·1	Third	0·02	Large swelling.
	9 Mar. ,,	0·01	—	—	—	—
	15 May ,,	0·02	0·02	Third	—	—
No. IX 11 Jan. 1911	13 Jan. 1911	—	2·5	Second	0·1	Almost nil.
	22 Feb. ,,	—	—	—	1·5	Death 5th day.
	24 Feb. ,,	—	0·5	Third	0·25	Death 10th day.
	9 Mar. ,,	0·0025	—	—	—	—
	6 Apr. ,,	—	0·5	Second	0·1	Death 5th day.
	20 Apr. ,,	0·005	—	—	—	—
	28 Apr. ,,	0·0025	0·5	Second	0·1	Death 8th day.
No. X 14 Mar. 1911	20 Mar. 1911	—	0·1	Second	—	—
	27 Mar. ,,	0·003	—	—	—	—
No. XI 12 Jan. 1911	18 Jan. 1911	—	2·5	Third	0·1	Lived.
	6 Apr. ,,	—	0·5	Fourth	0·1	Death 7th day.
	28 Apr. ,,	0·003	0·5	Second	0·1	Lived.
	15 May ,,	0·003	0·1	Third	—	—
No. V 25 Feb. 1911	6 Apr. 1911	—	0·5	Second	0·1	Lived.
	20 Apr. ,,	0·0075	2·5	Fourth	0·5	Lived.
	28 Apr. ,,	0·0025	0·5	Second	0·1	Death 5th day.
No. VI 25 Feb. 1911	11 Mar. 1911	—	2·5	Second	0·1	Lived.
	6 Apr. ,,	—	0·1	Fourth	—	—
	20 Apr. ,,	0·005	—	—	—	—
	28 Apr. ,,	0·0025	0·1	Second	—	—
No. VII 7 Dec. 1910	10 Dec. 1910	—	2·5	Second	0·1	Lived.
	14 Dec. ,,	—	—	—	2·0	Lived.
	5 Apr. 1911	—	2·5	Second	0·5	Lived.
	21 Apr. ,,	0·005	—	—	—	—
	15 May ,,	0·003	0·1	Third	—	—
	15 May ,,	—	0·5	Fourth	—	—
No. VIII 7 Dec. 1910	12 Dec. 1910	—	2·5	Second	0·1	Lived.
	5 Apr. 1911	—	2·5	Second	0·5	Death 5th day.
	21 Apr. ,,	0·005	—	—	—	—

TABLE II.

*Observations on different strains on the same date showing that the broth used was not the cause of differences in virulence.*

Strain		Date of experiment	Deposit from 10 c.c. of culture	M.L.D., whole culture	Day of death	Deposit from 10 c.c. of emulsion	M.L.D., original emulsion of bacilli	Day of death	Sublethal dose original emulsion	M.L.D. standard emulsion	Day of death	Sublethal dose standard emulsion
No.I	15 May'11	.005	0.02	Second	0.009	0.02	Second	—	0.02	Second	—	
No.II	"	.02	0.02	Third	0.01	0.1	Third	0.02	0.1	Third	0.02	
No.XI.	"	.003	0.1	Third	0.015	0.33	Third	0.066	0.5	Third	0.1	
No.VII.	"	.003	0.1	Third	0.003	1.5	Third	0.3	0.5	Third	0.1	
B.												
Strain		Date of experiment	M.L.D., whole culture	Day of death	Sublethal dose	Strain	Date of experiment	M.L.D., standard emulsion	Day of death	Sublethal dose		
No. VII	5 Apr. 1911	2.5	Second	0.5	No. I	6 Mar. 1911	0.01	Second	—	—		
No. XII	"	0.5	Fourth	0.1	No. XI	"	4.0	Third	1.0			
No. XIII	"	0.1	Second	—								
No. VIII	"	2.5	Second	0.5								

The variation in deposit for the same strain was not proportionate to the changes in pathogenicity, e.g. strain V on the occasion on which the deposit was largest (0.0075 c.c.) had a M.L.D. of 2.5 c.c., whereas on another occasion with a smaller deposit of 0.0025 to 0.003 c.c. the M.L.D. was actually smaller, viz. 0.5 c.c.

Strain XI twice gave a deposit of 0.003 c.c. but on one occasion the M.L.D. was 0.5 c.c. and on the other 0.1 c.c.

It appears, therefore, that neither in regard to different strains, nor to different observations on the same strain, were the comparatively small variations in the amount of deposit proportional to the pathogenicity of the culture.

The difference in pathogenicity between the strains was much greater than could be accounted for by the difference in the amount of growth in the respective cultures.

Although the degree of pathogenicity of the same strain varied from time to time, the difference between the strains for the most part remained well marked.

*The independent variation of Bacilli and Toxin as regards  
Lethal power in two-day broth cultures.*

Workers who have investigated the relation of virulence to toxigenicity of different strains of *B. diphtheriae* have usually made use of 24-hour broth or young agar cultures in estimating the virulence, and filtrates from broth cultures of about a week old, for testing the amount of toxin produced. They have come to the conclusion that the same bacillus may be highly virulent and yet of feeble toxigenic power or the reverse (Martin, L., 1898, Behring, 1901). In the tests recorded in the present communication the virulence and toxigenicity have both been examined by means of a two-day broth culture.

It seemed that from the point of view of virulence it was of more interest to know the relation, as regards lethal power, between the toxin and bacilli in the same culture. Moreover it seemed unlikely that the amount of toxin accumulated in a 7-day broth culture had any very intimate relation to the pathogenicity of a particular strain, as the experimental animal injected with bacilli or culture usually dies within three days. Also the exact composition of the broth probably has a special effect on the accumulation of toxin independently of its formation.

*Methods of testing the Toxigenicity and Virulence.*

A measured quantity of the culture was centrifuged and the clear fluid poured off and re-centrifuged. The resulting clear fluid was tested for its toxin-content.

The deposit after the first centrifuging was washed with salt solution, and after again centrifuging the deposit was made up to the original volume. The M.L.D. of the resulting bacterial emulsion was taken as a criterion of "Virulence proper."

The doses of toxin or bacterial emulsion employed were 0·01, 0·02, 0·1, 0·5 and 2·5 c.c. and occasionally doses with smaller intervals were used.

In order to reduce the dose of bacilli to a uniform measure a standard bacillary emulsion was used of such a strength that 10 c.c. of emulsion, on centrifuging, yielded a deposit of 0·01 c.c. of bacilli. The M.L.D. of bacilli could then be stated as a dose of such a standard emulsion. In a few instances a standard emulsion was actually made, and used for

injecting the animals, but usually the original emulsion was used and the dose of standard emulsion was calculated from the amount of deposit given by 10 c.c. of the original emulsion.

In Table III the M.L.D. of the original emulsion of washed bacilli made up to the same volume as the culture, and of the standard emulsion are shown, and the amount of deposit from 10 c.c. of the emulsion used is also shown.

TABLE III.

Strain and date of isolation	Date of experi- ment	Deposit from 10 c.c. of emul- sion	M.L.D. of origi- nal emulsion	Day of death	Sublethal dose	Result of do.	M.L.D. of stand- ard emulsion	Sublethal dose of standard emul- sion	Result of do.
No. I 24 Jan. '11	16 Feb. '11	—	0·01	Third	—	—	—	—	—
	6 Mar. ,,	0·01	0·01	Second	—	—	0·01	—	—
	20 Mar. ,,	—	0·01	Third	—	—	—	—	—
	15 May ,,	0·009	0·02	Second	—	—	0·02	—	—
No. II 2 Feb. '09	9 Dec. '09	—	0·1	—	—	—	—	—	—
	3 Feb. '11	—	0·1	Third	0·02	Lived	—	—	—
	9 Mar. ,,	0·01	2·0	Second	0·5	Lived	2·0	0·5	Lived.
	15 May ,,	0·01	0·1	Third	0·02	Lived	0·1	0·02	Lived.
No. IX 11 Jan. '11	24 Feb. '11	—	>1·0	—	1·0	Death 10th day	—	—	—
	9 Mar. ,,	0·01	0·1	Fourth	0·02	Lived	0·1	0·02	Lived.
	20 Apr. ,,	0·005	0·5	Fourth	0·1	Lived	0·17	0·03	Lived.
No. X 14 Mar. '11	27 Mar. '11	0·004	0·05	Fourth	—	—	0·02	—	—
No. XI 12 Jan. '11	10 Feb. '11	—	>3·0	—	3·0	Lived	—	—	—
	20 Apr. ,,	0·0075	2·5	Third	0·5	Lived	1·5	0·3	Lived.
	6 Mar. ,,	—	—	—	—	—	4·0	1·0	Lived.
	15 May ,,	0·015	0·33	Third	0·066	Lived	0·5	0·1	Lived.
No. V 25 Feb. '11	20 Apr. '11	0·003	0·5	Fourth	0·1	Lived	0·17	0·03	Lived.
No. VI 25 Feb. '11	16 Mar. '11	—	>4·0	—	4·0	Lived	—	—	—
	20 Apr. ,,	0·003	—	Second	0·1	Lived	0·17	0·03	Lived.
No. VII 6 Dec. '10	21 Apr. '11	0·003	>2·5	Seventh	2·5	Death 7th day	>0·83	0·83	Death 7th day.
	15 May ,,	0·003	1·5	Third	0·3	Lived	0·5	0·1	Lived.
No. VIII 7 Dec. '10	21 Apr. '11	0·005	2·5	Third	0·5	Lived	1·25	0·25	Lived.

Table III shows that the M.L.D. of standard emulsion varied for the same strain at most from 0·1 to 2·0 c.c. in the case of No. II, but so large a M.L.D. as 2 c.c. was quite unusual for this strain. Leaving this one observation out of consideration the greatest variation for a single strain was in the case of No. XI which on different occasions gave a M.L.D. of 4 c.c. and 0·5 c.c. (variation of 8 to 1). The differences amongst the different strains, if one takes the occasions of least difference, ranged from 0·5 to 0·01 (50 to 1), or if one takes the greatest differences, from 4 to 0·01 (400 to 1).

The nine strains fall roughly into three groups as regards their virulence as tested by the M.L.D. of standard emulsion.

- (1) Two strains, I and X, had a M.L.D. of 0·01 to 0·02 c.c.
- (2) Three strains, IX, V and VI, had a M.L.D. of 0·1 to 0·17 c.c.
- (3) Three strains, XI, VII and VIII, whose M.L.D. was 0·5 c.c. or more.

Strain II, whose M.L.D. varied from 2·0 to 0·1 c.c., should probably be included in the second group; this strain had been tested for pathogenicity on several previous occasions and the M.L.D. was never more than 0·1 c.c. The occasion on which the M.L.D. of original emulsion and standard emulsion was 2·0 c.c. was altogether exceptional.

The M.L.D. of standard emulsion and of original emulsion of the same strain were not widely different when they were both examined on the same occasion. This is merely another way of stating that the differences in deposit yielded by the original emulsions of the different strains were not very large, and further that variations in the inherent properties of the bacillus were of more importance when determining the M.L.D. than variations in the number of bacilli present.

There is no evidence to show whether the greater virulence of some strains is due to increased resistance to the destructive powers of the animal body, greater rapidity of multiplication in the body or a greater ability to make toxin from the body fluids.

In Table IV the M.L.D. of toxin and that of the emulsion of washed bacilli made up to the original bulk of culture after centrifugalising, are shown together. The ratios of the minimal lethal doses of original emulsion and of the standard emulsion to the M.L.D. of toxin occurring in the same culture, are given in order to show the relative values of the bacilli and toxin as lethal agents in two-day cultures.

The ratio of the M.L.D. of the bacilli to that of the toxin obtained from the same culture was most commonly as 1/1; this was the case for 7 out of 11 strains examined, and shows that the lethal power of the

toxin and bacilli in a two-day culture are usually equal. This relation was curiously maintained, even when the same strain yielded cultures of high and low pathogenicity on different occasions, as in the case of strain VI.

TABLE IV.

*Comparison of the lethal power of washed bacilli and toxin in the same cultures (two days' growth).*

Strain	Date of experiment	M.L.D. of original emulsion	M.L.D. of standard emulsion	M.L.D. of toxin	Ratio of M.L.D. of original emulsion to M.L.D. of toxin	Ratio of M.L.D. of standard emulsion to M.L.D. of toxin
No. I	11 Feb. 1911	0·01	0·01	0·5	1/50	1/50
, II	3 Feb. ,	0·1	—	0·1	1/1	—
, III	9 Mar. ,	2·0	2·0	0·5	1/0·25	1/0·25
, IV	21 Dec. 1909	0·1	—	0·1	1/1	—
, V	21 Dec. ,	0·1	—	0·1	1/1	—
, VI	20 Apr. 1911	0·5	0·17	2·5	1/5	1/15
, VII	16 Mar. ,	4·0	—	4·0	1/1	1/3
, VIII	20 Apr. ,	0·5	0·17	0·5	1/1	1/3
, IX	21 Apr. ,	>2·5	>0·83	2·5	1/1	1/3
, X	9 Mar. ,	2·5	1·25	2·5	1/1	1/2
, XI	20 Apr. ,	0·1	—	1·0	1/10	—
, XII	27 Mar. ,	0·5	—	2·5	1/5	—
, XIII	10 Feb. ,	0·05	0·02	1·0	1/20	1/50
, XIV	20 April ,	>3·0	4·0	3·0	1/1	1/0·75
, XV	20 April ,	2·5	—	2·5	1/1	—

On the other hand the M.L.D. of bacillary emulsion of strains I, V, IX and X was smaller than that of the toxin, the ratio  $\frac{\text{M.L.D. bacilli}}{\text{M.L.D. toxin}}$  being represented by the fractions  $\frac{1}{50}$ ,  $\frac{1}{5}$ ,  $\frac{1}{10}$  or  $\frac{1}{3}$  and  $\frac{1}{20}$  respectively, so that, e.g. in the case of strain I, the bacilli in the culture had actually fifty times the lethal power of the toxin.

When the M.L.D. of standard emulsion is compared in the same way with the M.L.D. of toxin, none of the strains showed an equal value of standard emulsion and toxin. In two cases, e.g. strains II and XI, the M.L.D. of standard emulsion of bacilli, was actually slightly greater than that of the toxin, but more commonly the M.L.D. of toxin was the greater. For example in the cases of strains VI, VII and VIII the ratio  $\frac{\text{M.L.D. of standard emulsion}}{\text{M.L.D. of toxin}}$  was equal to  $\frac{1}{3}$ ,  $\frac{1}{3}$  and  $\frac{1}{2}$  respectively, and in strains I and X the ratio was represented by  $\frac{1}{50}$  in each case.

This method of comparison emphasises the fact that in a two-day culture the lethal value of the bacilli equals or may greatly exceed that

of the toxin, and that variation in the pathogenicity of the whole culture depends on the bacilli more than on the toxin.

Some strains, therefore, were apparently far better producers of toxin in the body than *in vitro*, perhaps on account of their greater resistance to the adverse influences met with in the body or on account of their more rapid multiplication there.

This is perhaps as good a criterion as can be found of their virulence proper, and such strains may be correctly described as of higher virulence than strains in which the toxin production *in vitro* and *in vivo* are more equal.

#### *Irregularities.*

The Minimal Lethal Dose of "whole culture," of toxin or of bacillary emulsion was not constant for individual strains, and this was not to be expected. There was, however, a very fair amount of agreement for most of the strains on the different occasions on which they were tested. Four strains of low virulence (Nos. V, VI, VII and XI) were retested after four passages through broth and in only one case (No. VI) did a smaller dose of the whole culture prove fatal.

The strains of low virulence which have been observed are widely separated from completely non-virulent strains. The difference may not, however, be much greater than that between strains of very high and very low virulence. The relation of the M.L.D. of a strain of very high virulence to one of low virulence may be expressed as a ratio of 1 to 50 or more. If the M.L.D. of a strain of low virulence bore the same ratio to the M.L.D. of a "non-virulent" strain, the latter would be lethal in a dose of 50 c.c. whereas a dose larger than 10 c.c. cannot be conveniently used.

#### *Incidental observations.*

In order to find out whether any considerable part of the lethal effect of injecting washed bacilli was due to toxin clinging to the bacilli, toluol (on other occasions chloroform) was added to the emulsion of washed bacilli and also to the separated toxin. They were then kept at room temperature for 24 to 48 hours till all the bacilli in the emulsion were dead. The M.L.D. of toxin was then found not to have altered materially but the bacilli had become almost innocuous, and 30 to 40 times the dose of bacillary emulsion, which when living caused death, now caused hardly any disturbance. With a similar object, a small

amount of antitoxin was added to the bacillary emulsion and the mixture incubated for half an hour and then centrifuged, the bacilli again washed and the emulsion made up to the original volume. The M.L.D. of bacilli remained about the same as that of the emulsion before the antitoxin was added.

It was thought that possibly strains of low virulence, or strains with no pathogenic action, in reality formed toxin but had acquired the property of destroying it with greater rapidity than ordinary virulent strains. Two-day cultures of non-virulent bacilli and virulent bacilli grown together were, however, almost as lethal as pure cultures of virulent bacilli.

#### CONCLUSIONS.

(1) The pathogenicity of different strains of *B. diphtheriae* when first isolated, as tested with two-day broth cultures, varies greatly (Minimal Lethal Dose varies as 400 to 1).

(2) There is a tendency for some strains of low virulence to increase slightly in pathogenicity in artificial culture, e.g. a fall of M.L.D. to  $\frac{1}{5}$ th of its original figure. The amount of growth in a two-day culture varies for different strains, but not in proportion to the lethal power of the culture.

(3) The pathogenicity of young two-day "whole" broth cultures of different strains of *B. diphtheriae*, even after the strains examined have been subjected to prolonged culture on artificial media, varies in a degree which may be expressed as 1 to 50.

(4) The virulence of washed bacilli from two-day broth cultures of different strains varies at least as much as the pathogenicity of whole cultures.

(5) The amount of growth in two-day cultures of different strains varies considerably but not in proportion to the pathogenicity of the culture.

(6) The M.L.D. of toxin in a two-day broth culture varies less for different strains than does the virulence of the bacilli in the same culture.

(7) The ratio of the M.L.D. of toxin in a two-day broth culture to that of the bacilli in the same culture may frequently be expressed as  $\frac{1}{2}$  or M.L.D. toxin = M.L.D. bacilli, but the M.L.D. of bacilli may be  $\frac{1}{50}$  that of the toxin. The M.L.D. of bacilli was only once observed to be greater than that of the toxin.

(8) The ratio of the M.L.D. of standard bacillary emulsion to the M.L.D. of toxin from the same culture as the bacilli, varies for different strains within about the same limits as the ratio of the M.L.D. of the original emulsion to that of the toxin. The M.L.D. of standard emulsion is, however, sometimes greater than that of the toxin.  $\frac{\text{M.L.D. standard emulsion}}{\text{M.L.D. toxin}}$   
 varies for different strains from  $\frac{1}{0.25}$  to  $\frac{1}{50}$ .

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## IMMUNITY OF GUINEA-PIGS TO DIPHTHERIA TOXIN AND ITS EFFECT UPON THE OFFSPRING.

### PART 2.

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#### C. *Immunity transmitted to young when both parents have been injected with various mixtures of toxin and antitoxin.*

IN Part 1 results of the injection of the mothers only have been recorded; we shall now deal with the young of parents which have both received an injection of a toxin-antitoxin mixture. When this work was commenced a limited number of injected pigs were isolated for breeding and their number added to from time to time during the course of these investigations. A complete history was kept of each individual and full notes were taken of all events connected with them from the time of the injection of the parents until the testing of the young of several litters. The number of pigs that could be thus kept apart and individually watched was of necessity strictly limited. In order to obtain easily statistical data that would give indications for subsequent work we kept all survivals from routine toxin-antitoxin injections in runs for breeding without any attempt to isolate individual pigs. The only separation was into two groups—Cage 79 for all pigs that had received injections near the L+ doses, *i.e.* those that had given large local reactions, and Cage 80 for those near the L0 doses, giving little or no reactions. In these runs were put all pigs surviving, whether bucks or does. The general scale of immunity among the young of these pigs was no higher than that among the young from normal bucks and injected does.

This agrees with the generally recognised fact that there is no transmission of immunity through the male.

Figures from some 160 young were obtained from these two groups and results are given here as a confirmation of certain results in Part 1.

*Higher Immunity conferred by L+ than by L0 doses.*

Higher immunity was obtained in the young from Cage 79 than in those from Cage 80, thus confirming results recorded in Tables I, II, III of Part 1. The figures are given in Tables XVIII, XIX and XX.

TABLE XVIII.

*Results of immunity of young divided according to the nature of the toxin-antitoxin mixture injected into the parents.*

		Toxin-antitoxin mixture injected into parents	
		L0	L+
Deaths	Under 0·008 c.c.	11	2
	0·008 c.c. and under 0·010 c.c.	20	9
	0·010   ,,   0·020   ,,	12	21
	0·020   ,,   0·030   ,,	6	7
Survivals	0·008 c.c. and under 0·010 c.c.	4	13
	0·010   ,,   0·020   ,,	21	22
	0·020   ,,   0·030   ,,	8	8
	Over 0·030 c.c.	1	1

TABLE XIX.

Dose at or above which young survived and at or under which young died	Toxin-antitoxin mixture injected into parents			
	L0		L+	
Survivals	Deaths	Survivals	Deaths	
0·008 c.c.	34	11	44	2
0·010   ,,	30	31	31	11
0·020   ,,	9	43	9	32
0·030   ,,	1	49	1	39

TABLE XX.

*Showing the percentage number of young surviving any particular dose.*

Dose	Toxin antitoxin mixture injected into parents	
	L0	L+
0·008 c.c.	75	95
0·010   ,,	49	73
0·020   ,,	17	21
0·030   ,,	2	2

As will be apparent, these tables differ in preparation from the corresponding ones in Part I since in the one case the mothers were all identified and it was possible to represent their immunity by the highest dose tolerated by, or the lowest dose fatal to, their young. In this way each mother was represented once in the tables. The tables now given are compiled from the degree of immunity shown by the individual young bred in one or other run containing L+ or L0 parents respectively. This difference in the tables is necessary because many of the young could not be identified with a particular mother and in no case was the father known as several bucks were present in each run.

From Table XX it will be seen that 73% of the young from L+ parents showed slight immunity (*i.e.* survived a dose of 0·010 c.c. toxin) while only 49% from L0 parents survived the same dose.

*Rate of loss of immunity in the young.*

Results of tests upon pigs of different ages (but of the same weight —250 grams) were tabulated and are given in Tables XXI, XXII and XXIII, corresponding to Tables IX, X and XI in Part I.

TABLE XXI.

		Age of young in days					
		20 or less	21-30	31-40	41-50	51-60	Over 60
Deaths	Under 0·008 c.c.	1	1	2	3	4	2
	0·008 c.c. and under 0·010 c.c.	3	9	5	5	2	5
	0·010	" "	0·020 "	0	4	6	9
	" "	0·030 "	2	1	4	2	2
Sur-	0·008 c.c. and under 0·010 c.c.	4	9	3	1	0	0
vivals	0·010	" "	0·020 "	5	13	10	10
	" "	0·030 "	1	7	5	1	0
	Over 0·030 c.c.	0	0	0	1	0	1

TABLE XXII.

Doses at or above which young survived and at or under which young died	Age of young in days											
	20 or less		21-30		31-40		41-50		51-60		Over 60	
	Sur-	Deaths	Sur-	Deaths	Sur-	Deaths	Sur-	Deaths	Sur-	Deaths	Sur-	Deaths
0·008 c.c.	10	1	29	1	18	2	13	3	3	4	5	2
0·010 ,	6	4	20	10	15	7	12	8	3	6	5	7
0·020 ,	1	4	7	14	5	13	2	17	0	12	3	15
0·030 ,	0	6	0	15	0	17	1	19	0	14	1	17

From Table XXIII it will be seen that the number of pigs surviving decreases with the increased age after 40 days from birth. The figures are not so regular as in Table XI because the numbers here are fewer. This is particularly the case with the pigs over 60 days old, three

TABLE XXIII.

*Showing the percentage number of young of different ages that survived any particular dose.*

Dose	Age of young in days					
	20 or less	21-30	31-40	41-50	51-60	Over 60
0·008 c.c.	90	96	90	81	42	71
0·010 „	60	66	68	60	33	41
0·020 „	20	33	27	10	0	16
0·030 „	0	0	0	5	0	5

exceptional pigs fully account for the irregularity of the figures. These pigs were recorded as three months old and yet showed considerable immunity. These cases are not specially emphasised as we cannot place absolute reliance upon individual results from these groups where the isolation and observation were not as rigid as in the pigs from which Part I results were obtained. If we assume that the rate of loss of immunity in the young is the same for the young of all injected does whether the bucks were normal or injected, then we can group the figures from both sets of pigs and obtain statistics from well over 500 pigs. These are given in Tables XXIV, XXV and XXVI. In all cases the pigs were tested directly they reached the weight of 250 grams.

From Table XXVI it will be seen that up to the age of 40 days at least 90% of young survived 0·008 c.c., from 41-50 days 88%, from 51-60 days 52%, and over 60 days 27%. The results in this table appear to

TABLE XXIV.

	Age of young in days					
	20 or less	21-30	31-40	41-50	51-60	Over 60
Deaths	Under 0·008 c.c.	3	5	4	4	11
	0·008 c.c. and under 0·010 c.c.	7	30	26	17	6
	0·010 „ „ 0·020 „	4	16	20	21	19
	0·020 „ „ 0·030 „	7	8	11	4	11
Survivals	0·008 c.c. and under 0·010 c.c.	13	19	7	2	1
	0·010 „ „ 0·020 „	13	30	28	19	6
	0·020 „ „ 0·030 „	2	14	15	5	5
	Over 0·030 c.c.		1	3	4	0

TABLE XXV.

Doses at or above which young survived and at or under which young died	Age of young in days											
	20 or less		21-30		31-40		41-50		51-60		Over 60	
	Survivals	Deaths	Survivals	Deaths	Survivals	Deaths	Survivals	Deaths	Survivals	Deaths	Survivals	Deaths
0·008 c.c.	29	3	66	5	54	4	30	4	12	11	6	16
0·010 ,,	16	10	47	35	47	30	28	21	11	17	6	22
0·020 ,,	3	14	17	51	19	50	9	42	5	36	3	36
0·030 ,,	1	21	3	69	4	61	4	46	0	47	1	43

TABLE XXVI.

*Showing the percentage number of young of different ages that survived any particular dose.*

Dose	Age of young in days					
	20 or less	21-30	31-40	41-50	51-60	Over 60
0·008 c.c.	90	93	92	88	52	27
0·010 ,,	61	57	61	57	39	21
0·020 ,,	17	25	27	17	12	7
0·030 ,,	4	2	6	2	0	2

be almost uniform with any age up to 40 days with the tendency towards a maximum immunity among pigs between 30 and 40 days old rather than among younger pigs. All individual cases recorded indicate definitely that in pigs of the same litter (or in all pigs from the same mother if allowance is made for the progressive loss of immunity in the mother) the youngest pig gives the highest immunity without any such optimum age as would be indicated in Table XXVI. This apparent discrepancy would seem to show that the same causes that tend to yield high immunity in the mother also tend to delay the rate of growth in the subsequent offspring. If this were the case the majority of exceptionally young pigs tested would be the offspring of pigs of less immunity and the first two columns of Table XXVI would refer to pigs of a lower grade of immunity than those of subsequent columns.

#### D. Evidence of lowered resistance in young of injected parents.

While testing young for traces of immunity it was noticed that in some cases the young from injected parents died earlier than the controls. A few isolated instances of earlier deaths would be of no significance because marked differences in the lethal time are fairly frequent in normal pigs injected on the same day. It was noticed, however, that in

the case of certain does a large majority of their young injected with normal lethal doses died earlier than the controls. In such a case one is forced to conclude that either the immunity of the mother has passed off, that the immunity transmitted to the young by the mother has disappeared during the growth of the pig or that the mother failed to produce antitoxin in response to the injection, and that the absence of passive immunity then brings into evidence the damaging action of the toxin-antitoxin injection or of some disturbing factor other than the toxin in the toxin-antitoxin mixture. It is also possible that certain strains of guinea-pigs possess naturally either a greater or less resistance than the normal. This while possibly accounting for certain irregularities, cannot explain such results as are given in Tables XXIX to XXXII.

In Cage 79 where the young were bred from parents that had received L+ doses, only one case was noticed in which the young showed apparently a lowered resistance. This is given in Table XXVII.

TABLE XXVII.

*Evidence of lowered resistance among young bred from L+ parents.*

Cage	Litter	Age of young	Treatment of young		Control pig	
			Dose	Result	Dose	Result
79 F	1	20 days	0·0085 c.c.	Large reaction increasing 10 grms. in weight	—	—
	2	55 ..	0·010 ..	Died in 3 days	0·010 c.c.	Died in 5 days
		62 ..	0·008 ..	" 4 "	0·008 ..	" 7 "
		62 ..	0·010 ..	" 3 "	0·010 ..	" 3 "

A 20-day old pig of the first litter easily survived a normal lethal dose. In the second litter where the pigs were 55 and 62 days old when tested two out of three died earlier than the controls. This result standing alone would be of very little significance but some marked instances occurred in Cage 80 recorded in Table XXVIII. The first litter of pig 80 CC was tested with too high a dose, but in the second litter one of the young died in five days after an injection of 0·005 c.c. when a control normal pig survived a higher dose (0·0055 c.c.). In the third litter all three of the offspring died earlier than the controls. Similar results were obtained with the 2nd, 3rd and 4th litters of 80 N. The greater susceptibility here shown cannot in every case be accounted for by the variation in resistance dependent on the age of the

pig; in 80 CC young of 28 days in one litter and 53 days in another showed lowered resistance while in Cage 80 N young of different litters 14 days and 53 days old died earlier than the controls.

A small summary of Table XXVIII will show that the lowering of resistance is quite definite. In Cage 80 CC four pigs are directly comparable with the controls, of these one died when control survived a higher dose and three died earlier than the normal pigs. In Cage 80 N of five pigs compared two died when controls survived the same dose and the other three died earlier.

It appears advisable to record here other evidence of lowered resistance in young bred from injected does (the case of the offspring of injected bucks and normal does will be dealt with later). Only a few instances were noted among the young of normal bucks and injected does and these are given in Table XXIX.

TABLE XXVIII.

*Evidence of lowered resistance among young bred from L0 parents.*

Cage	Litter	Age of young	Treatment of young		Control pig	
			Dose	Result	Dose	Result
80 CC	1	49 days	0·016 c.c.	Died in 3 days	0·007 c.c.	Died in 4 days
		49 "	0·012 "	" 2 "	0·007 "	" 4 "
		49 "	0·008 "	" 3½ "	0·007 "	" 4 "
	2	48 "	0·0055 "	" 3 "	0·006 "	" 3 "
		53 "	0·0050 "	" 5 "	0·0055 "	Survived
	3	28 "	0·007 "	" 6 "	0·007 "	Died in 7 days
		28 "	0·008 "	" 4 "	0·008 "	" 6 "
		35 "	0·008 "	" 4 "	0·008 "	" 11 "
					0·008 "	" 7 "
80 N	1	21 "	0·016 "	" 7 "	...	...
		24 "	0·017 "	" 3 "	...	...
	2	49 "	0·008 "	" 4 "	0·0065 "	Died in 4 days
		49 "	0·012 "	" 3 "		
		53 "	0·007 "	" 4½ "	0·007 "	Survived
	3	41 "	0·0055 "	" 6 "	0·0055 "	Died in 8 days
		52 "	0·006 "	" 5 "	0·006 "	Survived
	4	14 "	0·007 "	" 4 "	0·007 "	Died in 5 days
		14 "	0·008 "	" 4 "	0·008 "	" 14 "

No instances were noticed among the young from normal bucks and does which had received L+ doses. The cases in Table XXIX were all from L0 does and it is decidedly significant that in each case the injected mixture contained at least  $\frac{1}{2}$  c.c. of horse serum. It would

appear that in the usual routine toxin-antitoxin injections, where the quantity of horse serum would average 1/500 c.c., those doses that would affect the mother sufficiently to lower the resistance of the offspring must contain sufficient uncombined toxoid to produce considerable immunity. The degree of lowering of resistance is very small compared with the degree of immunity conferred and so it is rare that lowered resistance can be demonstrated. The effect is presumably non-specific and so could probably be shown if the young were tested

TABLE XXIX.

*Evidence of lowered resistance among young bred from normal bucks, and does which had been injected with toxin-antitoxin mixtures.*

Cage	Litter	Age of young	Treatment of young		Control pig	
			Dose	Result	Dose	Result
106 P	1	45 days	0·015 c.c.	Died in 2 days	0·0065 c.c.	Died in 5 days
	2	66 "	0·015 "	" 2 "	0·006 "	" 3 "
		80 "	0·006 "	" 2 "	0·006 "	" 5 "
		82 "	0·0055 "	" 3 "	{ 0·0055 "	Survived
					{ 0·006 "	Died in 5 days
127 L	1	58 "	0·015 "	" 3 "	0·006 "	" 5 "
		66 "	0·006 "	" 5 "	0·006 "	Survived
					0·0065 "	Died in 8 days
	2	28 "	0·008 "	" 3 "	{ 0·008 "	" 5 "
		28 "	0·008 "	" 4 "	{ 0·008 "	" 5 "
		31 "	0·008 "	" 5 "	{ 0·008 "	" 5 "
					{ 0·008 "	11 "
105 BB	1	25 "	0·012 "	" 3 "	0·006 "	Survived
	2	35 "	0·008 "	" 3 "	{ 0·008 "	Died in 5 days
		35 "	0·008 "	" 4 "	{ 0·008 "	" 5 "
		52 "	0·008 "	" 3 "	0·008 "	" 4 "

against a toxin other than that to which they are passively immune. A few pigs bred from mothers highly immune to diphtheria toxin were tested against living typhoid organisms but no reliable results were obtained owing to the great range of inaccuracy. It follows that, in dealing with young bred from normal bucks and injected does, lowered resistance can only be demonstrated in those cases where the effect on the general vitality of the mother is due to causes other than a simple toxin-antitoxin mixture, such as  $\frac{1}{2}$  c.c. of horse serum as in the cases under consideration. It is possible that the cases in Tables XXVII and XXVIII were of does which had received injections containing large quantities of horse serum. It should again be stated that Tables XXVII

and XXVIII refer to pigs from cages 79 and 80 where the individual treatment was unknown.

In the course of experiments as to whether passive immunity in the mother could be transmitted to the young, various doses were injected with 5 c.c. (5000 units) diphtheria antitoxic serum, before and during pregnancy, and again immediately after birth. The number of young surviving was not large and the results of all those tested for one lethal dose are recorded in Tables XXX, XXXI, XXXII.

TABLE XXX.

*Evidence of lowered resistance among young bred from normal bucks, and does which had been injected with antitoxic serum alone.*

Cage	Litter	Age of young	Treatment of young		Control pig	
			Dose	Result	Dose	Result
133	1	73 days	0·007 c.c.	Died in 7 days	0·007 c.c.	Survived
151	1	Died before reaching standard weight				
	2	69 days	0·006 c.c.	Survived, lost 65 grms.	0·006 ,	Survived, lost 5 grms.
	3	43 ,,	0·008 ,,	Died in 4 days	0·008 ,,	Died in 4 days
		50 ,,	0·008 ,,	" 4 "	0·008 ,,	" 5 "
					10·008 ,,	" 5 "

TABLE XXXI.

*Evidence of lowered resistance among young from normal does injected with antitoxic serum during pregnancy.*

Cage	Litter	Age of young	Treatment of young		Control pig	
			Dose	Result	Dose	Result
78	1	36 days	0·0085 c.c.	Died in 5 days	...	...
		59 ,,	0·013 "	" 3 "	0·013 c.c.	Died in 3 days
120	1	44 ,,	0·014 "	" 3 "	0·014 ,,,	,, 5 ,,
		44 ,,	0·014 "	" 5 "		
		44 ,,	0·015 "	" 4 "		
121	1	22 ,,	0·014 "	" 4 ,,	...	...
		22 ,,	0·016 "	" 4 ,,	...	...
		23 ,,	0·014 "	" 3 ,,"	0·014 c.c.	Died in 8 days

In each table the young appear slightly less resistant than normal pigs. In all cases the conditions of breeding and growth of the young were the same as those for the normal control pigs except the injection of serum into the mother. If the resistance of these pigs was quite

TABLE XXXII.

*Evidence of lowered resistance among young suckled by normal mothers injected with antitoxic serum immediately after birth of young.*

Cage	Litter	Age of young	Treatment of young		Control pig	
			Dose	Result	Dose	Result
82	1	39 days	0·008 c.c.	Died in 2 days	0·008 c.c.	Died in 5 days
84	1	18 ,,	0·008 ,,	" 3 ,,"	0·008 ,,"	" 8 ,,"
		20 ,,"	0·008 ,,"	" 3 ,,"	..."	..."
122	1	22 ,,"	0·014 ,,"	" 3 ,,"	0·013 c.c.	Died in 3 days
		25 ,,"	0·013 ,,"	" 7 ,,"	0·015 ,,"	" 3 ,,"
123	1	24 ,,"	0·014 ,,"	" 3 ,,"	0·013 ,,"	Survived
		35 ,,"	0·014 ,,"	" 3 ,,"	0·016 ,,"	" 3 ,,"

normal we could expect that the majority would die in the same time as controls injected with the same dose, a few would die earlier, a few later. A summary of the results from the three tables shows that 14 young from injected mothers can be compared directly with normal control animals, that none died later than the controls, six died in the same time, five died earlier, two died when the control lived and one survived but lost 65 grams in weight where the control lost only 5 grams. Thus from 14 young from injected mothers, eight showed lowered resistance. It would appear that in each case where the vitality of the mother was slightly affected by the injection of a foreign protein the resistance of the young to diphtheria toxin was lowered to a slight extent.

#### E. Active immunity conferred by single injections of toxin in pigs that have been rendered passively immune by maternal transmission.

That the immunity conferred on the young of immune does is not transmitted to the next generation has been shown by all investigators who have taken up the subject of the transmission of immunity from parents to their offspring. This is in accord with the fact that the immunity so conferred is of a passive kind and is lost well before the age at which pigs commence to breed. Our own experiments were performed with five first-generation pigs from mothers whose other offspring had shown good immunity. These pigs were paired with normal bucks and the young tested as soon as they had attained the weight of 250

grams; neither immunity nor increased susceptibility was shown, all died within a short time of the controls, three slightly earlier, three at the same time and three slightly later.

We have already seen in Part 1 Section B that sublethal doses of toxin (without antitoxin) injected into normal pigs produce no appreciable immunity in their young. The following results show that a single injection of toxin into a doe rendered passively immune by maternal transmission may result in the young of such an animal exhibiting a considerable degree of immunity.

An example may be recorded of a doe in Cage 79S<sub>2</sub> bred from parents that had both received L+ doses. This pig was born 19. iv. '08 and on 1. vi. '08 when 250 grams in weight was injected with 0·0095 c.c. toxin, there was no local reaction and no change in weight. A control normal pig injected with the same dose on the same day died in seven days. The doe was paired with a normal buck on 29. vii. '08 and gave birth to two young on 4. xii. '08, to still-borns on 15. iii. '09 and 21. v. '09, and to three young again on 6. viii. '09. The results of the tests upon the young were as follows.

Born 4. xii. '08.	Tested when 33 days old.	0·0055 c.c.	Large reaction and increased 20 grms. in weight.
" "	" "	0·010 "	Large reaction and lost 10 grms. in weight.
	Control pig	0·0055 "	Died in 7 days.
Born 6. viii. '09.	Tested when 25 days old.	0·008 "	Very large reaction, increased 25 grms. in weight.
" "	" "	0·015 "	Very large reaction, lost 25 grms. in weight.
" "	" "	0·030 "	Died in 4 days.
	Control pig	0·007 "	" 5 "

In this case an approximate normal lethal dose only was injected into the immune pig of the first generation, with the result that considerable immunity was conferred on the young of the second generation.

Contrasted with this case is that of a doe in Cage 79 P<sub>1</sub>, also bred from parents that had both received L+ doses. This pig was born 1. v. '08 and on 4. vi. '08 when 250 grams in weight was injected with 0·012 c.c. toxin. There was a large local reaction and a loss of 40 grams in weight. A control normal pig was injected with 0·011 c.c. toxin and died in 9 days. The doe was paired with a normal buck on 8. i. '09 and young were born 25. v. '09 and 17. viii. '09. The results of the tests upon the young were as follows.

*Immunity to Diphtheria Toxin*

Born 25. v. '09.	Tested when 14 days old.	0·008 c.c.	Very large reaction, lost 30 grms.
" " "	" 2 Controls	"	40 "
" " "	" Both died in 5 days.	"	"
Born 17. viii. '09.	Tested when 45 days old.	0·010 c.c. 98 B <sup>1</sup> .	Very large reaction, lost 65 grms.
" " "	" Control	0·016, 0·020, 0·016, 0·020	Died in 3 days.
" " "	" "	" "	3 "
" " "	" "	" "	7 "
" " "	" "	" "	4 "

In this case second generation young only 14 days old showed very slight immunity, while older pigs of the next litter showed distinct evidence of lowered resistance.

The main difference between the two cases was the degree to which the mother was affected by the dose of toxin. Little or no immunity was transmitted when the mother was severely affected, while there was considerable immunity in the young whose mother easily tolerated the dose of toxin given. Similar results were noted in many instances and these are recorded in detail in Tables XXXIII, XXXIV and XXXV, divided according to the degree to which the mother was affected.

In Table XXXIII are recorded results of tests upon the young of seven immune does, all of which easily survived the dose given. In all cases the young showed decided immunity. As much as 10 fatal doses of toxin jar 967 A was tolerated by the young of 164 L<sub>2</sub>.

Table XXXIV gives the results for eight does moderately affected by the toxin injected. Two cases, 164 S and 153 S, show little or no immunity.

The size of the local reaction and the change in weight are only general indications of the degree to which a pig is affected by an injection. The changes in weight recorded are those that take place in five days after the injection, a big decrease in weight (25 grams or more) or an increase are significant, but the

<sup>1</sup> The supply of test toxin 98 A, with which all the earlier tests were carried out, ran short in August 1909, and toxin 98 B, and in a few instances 967 A, was then used. In all results given so far no toxin number has been recorded because 98 A was used throughout, in all subsequent tables the toxin number is given. The relative values of the toxins used are as follows:

Original M.F.D. 98 A and B under 0·003 c.c.	{ Average M.F.D. when used 98 A, 0·008 c.c.
Original M.F.D. 967 A 0·002 c.c.	" " " " 98 B, 0·014 ,
	" " " " 967 A, 0·004 ,

98 A and B were two bottles filled at the same time with the same toxin prepared in 1900, 967 A was prepared in March 1909. The L<sub>0</sub> doses were similar, that of 98 A 0·27 c.c. and that of 967 A 0·32 c.c.

TABLE XXXII.

*Results of second generation tests when immune mothers easily survived the toxin injected.*

Cage	Injection of mother		Time elapsing between injection of mother and birth of young	Age of young	Injection of young		Control pig			
	Dose (c.c.)	Reaction			Dose (c.c.)	Toxin	Died in 5 days	Result		
					+ 10	+ 20				
100 J	0·010	Nil		30 days	0·012	98 A	Nil + 25	0·0055		
			59 "	"	0·020	"	Died in 7 days	0·0060		
			10 "	"	0·016	"	Large - 10	0·0070		
79 S <sub>2</sub>	0·0095	Nil	No change	6 "	0·0055	"	Large + 20	0·0055		
				33 "	0·010	"	" - 10	"		
				25 "	0·0080	"	Very large + 25			
				0·015	"	"	" - 25			
				0·030	"		Died in 4 days	0·0070		
127 BB	0·009	Trace	+ 25 grms.	10 "	0·016	98 B	Very large + 15	0·016		
94 NN	0·016	Large + 20	+ 20 "	3 "	0·020	"	Trace + 5	0·016		
				21 "	0·030	"	Very large + 10	0·016		
94 BB	{ 0·016 0·020 <sup>2</sup>	Very large	+ 45 "	14 "	0·040	"	- 5	...		
			+ 40 "	"	"	"	"	...		
164 L <sub>2</sub>	0·024	"	+ 40 "	9 "	0·030	"	- 5	...		
				35 "	0·030	"	- 15			
				35 "	0·050	"	- 55			
				35 "	0·040	967 A	" - 5	0·004		
94 C	0·020	Large	+ 10 "	5 "	0·006	98 A	Nil + 15	0·006		
				35 "	0·010	"	Trace + 5	0·006		
				21 "	0·009	"	Medium + 20	0·009		
				12 "	0·009	"	Medium + 30	0·009		
				31 "	0·009	"	Large + 15	0·008		
				31 "	0·018	"	Small + 10	0·008		

<sup>1</sup> In this and subsequent tables under column headed "Result" is given size of local reaction and number of grams gained or lost in weight during 5 days after the injection.

<sup>2</sup> A second injection given 10 days after the first.

## Immunity to Diphtheria Toxin

TABLE XXIV.

*Results of second generation tests when immune mothers were moderately affected by toxin injected.*

Case	Injection of mother			Time elapsing between injection of mother and birth of young			Injection of young			Control pigs		
	Dose (c.c.)	Reaction	Change of weight	Age of young	Dose (c.c.)	Toxin	Dose (c.c.)	Result	Dose (c.c.)	Result	Dose (c.c.)	Result
164 L <sub>4</sub>	0.040	Very large	No change	9 " 34 days	0.020	98 B	Large +20	" -15	0.016	Died in 7 days	...	...
79 B <sub>1</sub>	0.010	Small	-25	12 " —	0.030	"	Very large +20	" -15	0.007	Died in 3 days	" 6 "	"
19 F <sup>7</sup>	0.010	Very large	-10	11 " 34 days	0.008	98 A	" +25	" +25	0.014	" 7 "	" 19 "	"
79 A <sub>3</sub>	0.015	Large	-5	16 " 41 "	0.020	98 B	Died in 6 days	" 2 days	0.016	" 7 "	" 7 "	"
79 B <sub>2</sub>	0.016	Very large	-15	48 " 46 "	0.020	"	Very large -15	Died in 2 days	0.016	" 4 "	" 4 "	"
164 S	0.022	Large	-5	52 " 46 "	0.020	967 A	" 2 "	" 30	0.0035	Very large +10	0.016	Died in 7 days
158 D	0.022	"	-5	60 " 49 "	0.020	967 A	" 15	" 15	0.0080	Very large -15	" 4 "	"
153 S	0.024	"	No change	32 " 32 "	0.020	98 B	Died in 3 days	Nil +10	0.0080	" 5 "	" 5 "	"
				21 " 28 "	0.040	"	Very large +20	" 5	0.018	" 7 "	" 7 "	"
				38 " 32 "	0.016	98 A	" 25	" 25	0.0088	" 5 "	" 5 "	"
				52 " 52 "	0.0080	"	Died in 4 days	" 5	0.0088	" 5 "	" 5 "	"
				57 " 57 "	0.0080	98 B	Very large -25	" 30	0.016	" 7 "	" 7 "	"
							Died in 3 days	" 30	0.0080	Died in 4 days	" 5 "	"

TABLE XXXV.

*Results of second generation tests when immune mothers were markedly affected by toxin injected.*

Cage	Injection of mother		Time elapsing between injecting of another and birth of young	Age of young	Injection of young		Control pig	
	Dose (c.c.)	Reaction			Dose (c.c.)	Toxin	Dose (c.c.)	Result
79 P <sub>1</sub>	0.012	Large	-40	11 months	14 "	"	0.008	98 A
					14 "	"	0.008	Very large -30 }
106 PP	0.018	Very large	-50	13 "	45 "	"	0.010	98 B
					45 "	"	0.016	Very large -30 }
					45 "	"	0.020	-65 }
132 R	0.020	" "	-35	7 "	23 "	"	0.009	"
					10 "	"	0.009	Died in 3 days
46 A	0.020	" "	-20	9 "	28 "	"	0.008	"
					38 "	"	0.008	"
127 LL	0.020	" "	-45	11 "	52 "	"	0.020	"
					56 "	"	0.008	"
					56 "	"	0.007	"
79 PP	0.020	" "	-25	6 "	42 "	"	0.008	"
					45 "	"	0.008	"
					10 "	"	0.007	"
153 Z	0.026	" "	-20	7 "	21 "	"	0.008	"
80 X	0.030	" "	-55	21 "	56 "	"	0.012	"
					56 "	"	0.016	"
					11 "	"	0.018	"

79 P <sub>1</sub>	0.012	Large	-40	11 months	14 "	"	0.008	98 A
					14 "	"	0.008	Very large -30 }
106 PP	0.018	Very large	-50	13 "	45 "	"	0.010	98 B
					45 "	"	0.016	Very large -30 }
					45 "	"	0.020	-65 }
132 R	0.020	" "	-35	7 "	23 "	"	0.009	"
					10 "	"	0.009	Died in 3 days
46 A	0.020	" "	-20	9 "	28 "	"	0.008	"
					38 "	"	0.008	"
127 LL	0.020	" "	-45	11 "	52 "	"	0.020	"
					56 "	"	0.008	"
					56 "	"	0.007	"
79 PP	0.020	" "	-25	6 "	42 "	"	0.008	"
					45 "	"	0.008	"
					10 "	"	0.007	"
153 Z	0.026	" "	-20	7 "	21 "	"	0.008	"
80 X	0.030	" "	-55	21 "	56 "	"	0.012	"
					11 "	"	0.016	"

79 P <sub>1</sub>	0.012	Large	-40	11 months	14 "	"	0.008	98 A
					14 "	"	0.008	Very large -30 }
106 PP	0.018	Very large	-50	13 "	45 "	"	0.010	98 B
					45 "	"	0.016	Very large -30 }
					45 "	"	0.020	-65 }
132 R	0.020	" "	-35	7 "	23 "	"	0.009	"
					10 "	"	0.009	Died in 3 days
46 A	0.020	" "	-20	9 "	28 "	"	0.008	"
					38 "	"	0.008	"
127 LL	0.020	" "	-45	11 "	52 "	"	0.020	"
					56 "	"	0.008	"
					56 "	"	0.007	"
79 PP	0.020	" "	-25	6 "	42 "	"	0.008	"
					45 "	"	0.008	"
					10 "	"	0.007	"
153 Z	0.026	" "	-20	7 "	21 "	"	0.008	"
80 X	0.030	" "	-55	21 "	56 "	"	0.012	"
					11 "	"	0.016	"

79 P <sub>1</sub>	0.012	Large	-40	11 months	14 "	"	0.008	98 A
					14 "	"	0.008	Very large -30 }
106 PP	0.018	Very large	-50	13 "	45 "	"	0.010	98 B
					45 "	"	0.016	Very large -30 }
					45 "	"	0.020	-65 }
132 R	0.020	" "	-35	7 "	23 "	"	0.009	"
					10 "	"	0.009	Died in 3 days
46 A	0.020	" "	-20	9 "	28 "	"	0.008	"
					38 "	"	0.008	"
127 LL	0.020	" "	-45	11 "	52 "	"	0.020	"
					56 "	"	0.008	"
					56 "	"	0.007	"
79 PP	0.020	" "	-25	6 "	42 "	"	0.008	"
					45 "	"	0.008	"
					10 "	"	0.007	"
153 Z	0.026	" "	-20	7 "	21 "	"	0.008	"
80 X	0.030	" "	-55	21 "	56 "	"	0.012	"
					11 "	"	0.016	"

79 P <sub>1</sub>	0.012	Large	-40	11 months	14 "	"	0.008	98 A
					14 "	"	0.008	Very large -30 }
106 PP	0.018	Very large	-50	13 "	45 "	"	0.010	98 B
					45 "	"	0.016	Very large -30 }
					45 "	"	0.020	-65 }
132 R	0.020	" "	-35	7 "	23 "	"	0.009	"
					10 "	"	0.009	Died in 3 days
46 A	0.020	" "	-20	9 "	28 "	"	0.008	"
					38 "	"	0.008	"
127 LL	0.020	" "	-45	11 "	52 "	"	0.020	"
					56 "	"	0.008	"
					56 "	"	0.007	"
79 PP	0.020	" "	-25	6 "	42 "	"	0.008	"
					45 "	"	0.008	"
					10 "	"	0.007	"
153 Z	0.026	" "	-20	7 "	21 "	"	0.008	"
80 X	0.030	" "	-55	21 "	56 "	"	0.012	"
					11 "	"	0.016	"

79 P <sub>1</sub>	0.012	Large	-40	11 months	14 "	"	0.008	98 A
					14 "	"	0.008	Very large -30 }
106 PP	0.018	Very large	-50	13 "	45 "	"	0.010	98 B
					45 "	"	0.016	Very large -30 }
					45 "	"	0.020	-65 }
132 R	0.020	" "	-35	7 "	23 "	"	0.009	"
					10 "	"	0.009	Died in 3 days
46 A	0.020	" "	-20	9 "	28 "	"	0.008	"
					38 "	"	0.008	"
127 LL	0.020	" "	-45	11 "	52 "	"	0.020	"
					56 "	"	0.008	"
					56 "	"	0.007	"
79 PP	0.020	" "	-25	6 "	42 "	"	0.008	"
					45 "	"	0.008	"
					10 "	"	0.007	"
153 Z	0.026	" "	-20	7 "	21 "	"	0.008	"
80 X	0.030	" "	-55	21 "	56 "	"	0.012	"
					11 "	"	0.016	"

79 P <sub>1</sub>	0.012	Large	-40	11 months	14 "	"	0.008	98 A
					14 "	"	0.008	Very large -30 }
106 PP	0.018	Very large	-50	13 "	45 "	"	0.010	98 B
					45 "	"	0.016	Very large -30 }
					45 "	"	0.020	-65 }
132 R	0.020	" "	-35	7 "	23 "	"	0.009	"
					10 "	"	0.009	Died in 3 days
46 A	0.020	" "	-20	9 "	28 "	"	0.008	"
					38 "	"	0.008	"
127 LL	0.020	" "	-45	11 "	52 "	"	0.020	"
					56 "	"	0.008	"
					56 "	"	0.007	"
79 PP	0.020	" "	-25	6 "	42 "	"	0.008	"
					45 "	"	0.008	"
					10 "	"	0.007	"
153 Z	0.026	" "	-20	7 "	21 "	"	0.008	"
80 X	0.030	" "	-55	21 "	56 "	"	0.012	"
					11 "	"	0.016	"

79 P <sub>1</sub>	0.012	Large	-40	11 months	14 "	"	0.008	98 A
					14 "	"	0.008	Very large -30 }
106 PP	0.018	Very large	-50	13 "	45 "	"	0.010	98 B
					45 "	"	0.016	Very large -30 }
					45 "	"	0.020	-65 }
132 R	0.020	" "	-35	7 "	23 "	"	0.009	"
					10 "	"	0.009	Died in 3 days
46 A	0.020	" "	-20	9 "	28 "	"	0.008	"
					38 "	"	0.008	"
127 LL	0.020	" "	-45	11 "	52 "	"	0.020	"
					56 "	"	0.008	"
					56 "	"	0.007	"
79 PP	0.020	" "	-25	6 "	42 "	"	0.008	"
					45 "	"	0.008	"
					10 "	"	0.007	"
153 Z	0.026	" "	-20	7 "	21 "	"	0.008	"
80 X	0.030	" "	-55	21 "	56 "	"	0.012	"
					11 "	"	0.016	"

79 P <sub>1</sub>	0.012	Large	-40	11 months	14 "	"	0.008	98 A
					14 "	"	0.008	Very large -30 }
106 PP	0.018	Very large	-50	13 "	45 "	"	0.010	98 B
					45 "	"	0.016	Very large -30 }
					45 "	"	0.020	-65 }
132 R	0.020	" "	-35	7 "	23 "	"	0.009	"

meaning of a small decrease is not clear unless the pig is kept under observation for a considerable time until it regains its normal rate of growth. A small decrease (5 or 10 grams) would indicate a severe reaction if the general conditions of time of year, feeding, etc. were conducive to rapid growth, and if the weight of the pig did not rise considerably for some time. On the other hand, if conditions are unfavourable for rapid growth, as in the winter time, a small decrease in weight in five days, followed immediately by a normal rise, indicates very little constitutional disturbance. Thus it follows that Table XXXIV may include pigs affected with markedly different degrees of severity and that if these pigs had been kept under close observation for a longer time after the injection, some would have been included in Table XXXIII and some in Table XXXV. The main comparison, therefore, must be made between Tables XXXIII and XXXV.

In Table XXXV results are given for the young of eight does, all of which were severely affected. Three only of these produced young which survived a normal lethal dose. These cases are as follows.

Cage 79 P<sub>1</sub>, 14 days old young of first litter survived, older pigs of a subsequent litter showed no immunity.

Cage 153 Z. Only one test made on a 21 day old pig.

Cage 80 X. Only one test made on an 11 day old pig.

The young from the other five does showed no trace of immunity whatever, on the contrary there was evidence of an increased susceptibility as shown in Cage 79 PP, where five out of six young died earlier than the controls injected with the same dose.

From the three cases where very young offspring survived the normal lethal dose it would appear that pigs of this group may transmit a trace of immunity to their young. This trace is rapidly lost and any effect on the vitality of the offspring due to the severity of the reaction caused by the injection of the parent is then exhibited by a condition of greater susceptibility to the injection of toxin.

From these results it would appear that immunity in the second generation depends considerably, if not entirely, upon the ease with which the first generation doe tolerated the toxin injected. This accords with the experience gained in the course of general work on immunisation in which it is found that the most effective response to the production of antibodies is obtained when the dose administered produces a moderate disturbance only; if this is much exceeded so that the animal is severely affected its capacity for producing antibodies in response to the injection is greatly impaired.

*F. Immunity in young of the third generation.*

The results of the preceding section indicate a possibility of producing very high immunity in young whose female ancestors have received suitable stimuli in each generation. It would also seem possible to produce young of very low resistance in a similar way. Experiments have been commenced with these ends in view and the results thus far obtained are recorded in Tables XXXVI, XXXVII, XXXVIII and XXXIX, where the history of each generation is given. The two second generation pigs surviving in Cage 79 P<sub>1</sub> recorded in Table XXXV were each paired with a normal buck and their young showed indications of lowered resistance. These are detailed in Tables XXXVI and XXXVII. The two other cases of third generation pigs given in Tables XXXVIII and XXXIX were those whose parents and grandparents easily survived the doses given (see Table XXXIII, Cage 94 C and Table XXXIV, Cage 158 D). These pigs showed considerable immunity, particularly in the case of Table XXXIX, where a pig 47 days old gained in weight after an injection of 0·060 c.c. jar 98 B. This dose represents nearly a  $\frac{1}{4}$  of the L0 dose, i.e. 50 binding units of toxin.

At the time of going to press another third generation result has been obtained confirming those of Tables XXXVIII and XXXIX. Two pigs in cage 223 LA survived and gained in weight after injections of 6 and 10 fatal doses of a fresh toxin. The male parents were normal in each generation and of the females the great-grandmother was injected with a toxin-antitoxin mixture near to the L+ dose causing a large local reaction and loss in weight. The grandmother survived 0·024 c.c. of toxin 98 A with a large increase in weight, the mother survived 0·030 c.c. 98 B with a slight loss in weight.

TABLE XXXVI.

Doe injected with an L+ mixture during 1907.

Paired with a similarly injected buck.



Doe born 1. v. '08, injected 4. vi. '08 with 0·012 c.c. 98 A. Large reaction, lost 40 grms. in weight. (Control injected with 0·011 c.c., died in 9 days.)

Paired with a normal buck.



Doe born 25. v. '09, injected 8. vi. '09 with 0·008 c.c. 98 A. Very large reaction, lost 30 grms. in weight. (Control injected with 0·008 c.c., died in 5 days.)

Paired with a normal buck.



Born 21. xii. '09, injected when 45 days old with 0·016 c.c. 98 B, died in 4 days. (Control injected with 0·016 c.c., died in 5 days.)

TABLE XXXVII.

Doe injected with an L+ mixture during 1907.

Paired with a similarly injected buck.



Doe born 1. v. '08, injected with 0·012 c.c. 98 A. Large reaction, lost 40 grms. in weight. (Control injected with 0·011 c.c., died in 9 days.)

Paired with a normal buck.



Doe born 25. v. '09, injected with 0·008 c.c. 98 A. Very large reaction, lost 40 grms. in weight. (Control injected with 0·008 c.c., died in 5 days.)

Paired with a normal buck.



Born 18. i. '10. Injected when 31 days old with 0·012 c.c. 98 B. Very large reaction, lost 45 grms. in weight. (Control injected with 0·014 c.c., lost 45 grms.)

Born 3. v. '10. Injected when 21 days old with 0·016 c.c. 98 B. Very large reaction, lost 80 grms. in weight. (Control injected with 0·018 c.c., lost 75 grms.)

Born 12. viii. '10. Injected when 25 days old with 0·012 c.c. 98 B. Died in 8 days.

"	"	"	"	25	"	0·018	"	"	"	4	"
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(Control injected with 0·018 c.c., died in 7 days.)

TABLE XXXVIII.

Doe injected with L0 dose 22. i. '08.  
Paired with a normal buck.



Doe born 29. v. '08, injected 6. vii. '08 with 0·020 c.c. 98 A. Large reaction and increase of 10 grms. in weight. (Control injected with 0·016 c.c., died in 3 days.)

Paired with a normal buck.



Doe born 20. vii. '09, injected 20. viii. '09 with 0·010 c.c. 98 A. Small reaction and increase of 10 grms. in weight.

Paired with a normal buck.



Born 8. iii. '10, injected when 35 days old with 0·030 c.c. 98 B. Very large reaction. No change in weight. (Control injected with 0·016 c.c., died in 7 days.)

Born 1. viii. '10, injected when 32 days old with 0·050 c.c. 98 B. Very large reaction. Lost 10 grms. in weight. (Control injected with 0·016 c.c., died in 3 days.)

Born 1. viii. '10, injected when 32 days old with 0·030 c.c. 98 B. Large reaction. Gained 15 grms. in weight. (Control injected with 0·016 c.c., died in 3 days.)

TABLE XXXIX.

Doe injected with an L0 mixture 15. ii. '08.  
Paired with a normal buck.



Doe born 28. viii. '08. Injected 1. ix. '08 with 0·022 c.c. 98 A. Large reaction. Lost 5 grms. in weight. (Control injected with 0·012 c.c., died in 3 days.)

Paired with a normal buck.



Doe born 21. v. '09. Injected 11. vi. '09 with 0·008 c.c. 98 A. No reaction. Gained 10 grms. in weight. (Controls injected with 0·008 c.c., died in 3 and 4 days.)

Paired with a normal buck.



Born 12. v. '10. Injected when 43 days old with 0·040 c.c. 98 B. Medium reaction. Gained 10 grms. (Control injected with 0·018 c.c., died in 6 days.)

Born 12. v. '10. Injected when 47 days old with 0·060 c.c. 98 B. Very large reaction. Gained 5 grms. (Control injected with 0·018 c.c., died in 7 days.)

Born 6. ix. '10. Injected when 24 days old with 0·040 c.c. 98 B. No reaction. Gained 30 grms. (Control injected with 0·020 c.c., died in 4 days.)

Born 6. ix. 10. Injected when 24 days old with 0·060 c.c., no reaction. Gained 40 grms. (Control injected with 0·020 c.c., died in 4 days.)

## SUMMARY.

(1) The young of parents both of which have been injected with an immunising mixture of diphtheria toxin and antitoxin, show immunity of the same order as that of young from similarly treated mothers and normal fathers.

(2) The injection of certain foreign substances into a female guinea-pig appears to have a direct effect on the offspring in diminishing their resistance to diphtheria toxin, shown equally well by the young of mothers injected, (*a*) before the attainment of sexual maturity, (*b*) during pregnancy, and (*c*) after birth during the period of lactation.

(3) A single injection of diphtheria toxin may give rise to a condition of active immunity (as tested by the resistance of the young) in guinea-pigs possessing hereditarily transmitted passive immunity. Should this injection of toxin give rise to great constitutional disturbance, the young may show lowered resistance, whereas, if it give rise to but slight constitutional disturbance, the young show a high degree of immunity. These effects appear to be accentuated if similar injections are repeated in the next generation.

## INFECTIVE METHAEMOGLOBINAEMIA IN RATS CAUSED BY GAERTNER'S BACILLUS<sup>1</sup>.

By A. E. BOYCOTT.

(*From the Pathological Department, Guy's Hospital, London.*)

IN the course of some experiments designed to test the influence of feeding with oleic acid on the blood<sup>2</sup>, in which rats were used, it was found that in a certain number of animals the blood was more or less brown. The brown colour was found to be due to the presence of methaemoglobin. It soon appeared that oleic acid had nothing to do with the matter since rats with brown blood were found among the control animals as well as in those which had been subjected to special feeding. The spleens of the affected rats were noticeably enlarged and from them were obtained pure cultures of an organism which afterwards proved to be Gaertner's bacillus. Inoculation of fresh rats with these cultures in appropriate doses produced in a proportion of animals all the symptoms and changes of the natural disease.

The spontaneous occurrence of methaemoglobinaemia in animals and the fact that the condition may be produced by artificial bacillary infection have not, as far as I am aware, been previously described. The affected rats, at any rate when a considerable proportion of the haemoglobin is changed in albino animals, present rather a striking picture. The tail, feet and ears, and to a less extent the whole surface of the body, look pale, livid and blue<sup>3</sup>, and the normal bright pink of

<sup>1</sup> Towards the expenses of this research grants were made by the British Medical Association and by the Royal Society.

<sup>2</sup> *British Medical Journal*, 5 November, 1910.

<sup>3</sup> The bacillus which turned canaries blue described by H. G. Wells some years since was, so the author informs me, entirely imaginary.

the eyes is changed to a pale brownish red. These changes are particularly noticeable if normal albino rats are put side by side with the affected animals, and in this way comparatively slight degrees of methaemoglobinaemia may be detected. They are however not very obvious if pigmented rats are used, nor is anything characteristic necessarily found on post-mortem examination since the methaemoglobin is soon reduced after death. Hence the condition may easily have been missed in the absence of any blood examination before death, though the chocolate-coloured blood of a blue rat during life is strange enough to attract immediate notice.

The condition was first noticed in May 1910, and between that date and January 1911 ten well-marked cases were found among about 1000 tame rats which were used in the laboratory. Along with these there occurred a number of rats which, though not showing methaemoglobinaemia, were found to be infected with Gaertner's bacillus; some of these were suffering from obvious general illness, some were anaemic, some were apparently normal as regards their health and blood condition. A certain number of rats found dead among the stock were also probably examples of the disease. For about nine months therefore a smouldering epidemic prevailed among the rats. These rats came from a number of different places and no connection could be traced between their place of origin and the prevalence of infection. Infected animals occurred pretty continuously among the various lots of rats which formed the constantly changing population of the animal houses. Repeated disinfection did not prevent the occurrence of fresh cases, and in November 1910 an entirely fresh animal house was brought into use without producing any effect. It was therefore improbable that infection was in all cases contracted by the rats after arriving at this laboratory, a conclusion substantiated by the discovery of infected animals among batches of rats which were killed and examined immediately after arrival. It appears therefore that infection with Gaertner's bacillus is, or was, widespread among the ordinary stocks of laboratory rats. In the present case no very great mortality was caused. Epidemics associated with acute fatal illness due to Gaertner's bacillus and killing most of the animals exposed to infection are however known among rats. Such an epidemic occurred among tame rats in this laboratory in 1908, and about the same time Bainbridge investigated a similar outbreak among wild rats in confinement<sup>1</sup>; he

<sup>1</sup> *Journal of Pathology*, Vol. xiii. (1909) p. 342.

has also recently (1911) met with another acute Gaertner epidemic with great mortality among tame rats. Petrie and Macalister<sup>1</sup> found organisms belonging to the Gaertner group in wild rats in Suffolk, and Bainbridge and O'Brien<sup>2</sup> have lately shown that epidemics among guinea-pigs may be due to Gaertner's bacillus as well as to *B. suispestifer*<sup>3</sup>. The rat is therefore probably an important reservoir of Gaertner's bacillus in this country—perhaps in part owing to the use of bacillary rat poisons which, as Bainbridge showed<sup>4</sup>, often contain or consist of that organism.

Methaemoglobinaemia is a well-known result of some forms of poisoning in man and animals (nitrites and allied bodies, aniline, chlorates, acetanilide, etc.). There have also occurred in man a series of cases of cryptogenetic cyanosis in which the blood is more or less brown. This condition may persist, more or less continuously, for years, and is usually associated with either diarrhoea or extravagant constipation. There is however no absolute justification for the term "enterogenous cyanosis" which is often used. In all the earlier cases the brown pigment in the blood was described as methaemoglobin, but, since the recognition of sulphaemoglobin<sup>5</sup>, the majority have been found to be due to the presence of that substance<sup>6</sup> and cases of true methaemoglobinaemia must be regarded as being decidedly rare. One such case was pretty fully investigated by Gibson and Douglas<sup>7</sup> who, having on one occasion found what appears to have been a typical *B. coli* in a blood culture, named the condition "microbic cyanosis." Methaemoglobinaemia also appears to occur occasionally in the ill-defined group of diseases of young children known as Buhl's and Winckel's diseases<sup>8</sup>. Most of these seem to be severe infections of one kind or another; in

<sup>1</sup> Reports to the Local Government Board on public health and medical subjects, New Series, No. 52 (1911), p. 59. Dr G. H. K. Macalister informs me that these organisms have since been identified as genuine Gaertner strains.

<sup>2</sup> *Journal of Pathology*, Vol. xvi. (1911) p. 145.

<sup>3</sup> *Journal of Hygiene*, Vol. x. (1910) pp. 231, 287.

<sup>4</sup> *Journal of Pathology*, Vol. xiii. (1909) p. 457.

<sup>5</sup> See T. W. Clarke and W. H. Hartley, *Journal of Physiology*, Vol. xxxvi. (1907) p. 62.

<sup>6</sup> S. West and T. W. Clarke, *Lancet*, 1907, Vol. i. p. 272; A. E. Russell, *Trans. Path. Soc.*, London, Vol. lviii. (1907) p. 177; W. E. Wynter, *Proc. Roy. Soc. Med.* Vol. i. 1908, Clinical Section, pp. 48, 197; T. W. Clarke and R. M. Curtis, *Medical Record*, Vol. lxxviii. (1910) p. 987.

<sup>7</sup> G. A. Gibson and C. C. Douglas, *Lancet*, 1906, Vol. ii. p. 72; *Quart. Journ. Med.* Vol. i. (1907) p. 29.

<sup>8</sup> Knöpfelmacher, in Pfaundler and Schlossmann's *Hand. d. Kinderheilkunde*, Vol. i. (1910) p. 386.

a recent case, in which methaemoglobinaemia was observed, Röthler<sup>1</sup> isolated a staphylococcus. It is however by no means established that these obscure cases of human methaemoglobinaemia are infective in origin.

(1) *Changes in the blood of naturally infected rats.*

(a) *Methaemoglobin.* The brown colour of the blood is undoubtedly due to methaemoglobin and no evidence of the presence of any other brown or yellow pigment was obtained. The band in the red, which alone is clearly visible in the presence of oxyhaemoglobin, occupied the same place (620–640) in the spectrum as in solutions of methaemoglobin prepared by any of the ordinary methods; it remained unaltered on saturating with carbon monoxide and immediately disappeared on reduction. It was therefore certainly not sulphaemoglobin<sup>2</sup>, in which the band which is conspicuous in the presence of oxyhaemoglobin lies more blueward (610–630), moves further blueward<sup>3</sup> on saturation with CO and is, for some time at any rate, unaltered by the addition of reducing agents.

The presence of methaemoglobin (or any other brown pigment) is best ascertained by examining the blood in the ordinary way with the Gowers-Haldane haemoglobinometer. Very small quantities, which would show nothing abnormal on spectroscopic examination, may be detected after saturation with carbon monoxide since the full pink tint of the pure CO-haemoglobin of the haemoglobinometer standard will not be realised. In examining such blood it is best to first saturate with CO<sup>4</sup>; the degree by which the sample fails to be as pink as the standard solution gives one a rough idea of the relative amounts of haemoglobin and methaemoglobin present. If a grain or two of sodium hydrosulphite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) are then added, the methaemoglobin is at once reduced to haemoglobin and this turned to CO-haemoglobin: the

<sup>1</sup> *Deutsche med. Woch.* 1911, p. 545.

<sup>2</sup> See T. W. Clarke and W. H. Hurtley, *Journal of Physiology*, Vol. xxxvi. (1907) p. 62; S. West and T. W. Clarke, *Lancet*, 1907, Vol. i. p. 272.

<sup>3</sup> As with the pigment which may appear in rabbit's blood in aniline poisoning (C. Price Jones and A. E. Boycott, *Guy's Hospital Reports*, Vol. LXIII. (1909) p. 313), and which may be sulphaemoglobin. In man aniline poisoning produces real methaemoglobinaemia.

<sup>4</sup> Owing to the possibility of the presence of cyanogen compounds in illuminating gas, it is best to use pure CO rather than coal gas to avoid difficulties of colour which might arise from the formation of cyanmethaemoglobin (see J. S. Haldane, *Journal of Physiology*, Vol. xxv. (1900) p. 230; A. S. Grünbaum, *ib.* Vol. xxxvi. (1907), *Proceedings*, p. iv).

percentage of haemoglobin in the sample is in this way readily ascertained. If, after reduction, the full pink of the standard cannot be obtained after repeated saturation with CO, it may be assumed that some brown pigment other than methaemoglobin is present.

The methaemoglobin is contained within the corpuscles; none is present in plasma or serum. No histological changes were found in the red cells which appeared to correspond to the presence of methaemoglobin, nor indeed do I know whether the change involved all the haemoglobin in some cells or some of the haemoglobin in all the cells. The proportion of haemoglobin changed was determined in but one spontaneous case; this animal was comparatively slightly affected and only 12 per cent. was methaemoglobin. In a number of inoculated rats however more than half the blood pigment was methaemoglobin, the highest figure observed being 68 per cent. (see Table IV below, p. 454). As might be expected, such animals showed marked dyspnoea.

After death the methaemoglobin is quickly reduced and disappears. In several cases in which the time of death of blue rats was accurately observed, the heart blood contained no methaemoglobin when the animal was examined as soon as one hour later. In no case was methaemoglobin present in affected rats which were found dead in the morning. As will be noted below, in many of the animals methaemoglobinaemia was associated with more or less profound anaemia and various forms of young red cells were abundantly present in the blood. It has been shown by Douglas<sup>1</sup> and by Morawitz and Pratt<sup>2</sup> that such anaemic blood consumes oxygen very much faster than normal blood, and that the effective agents are immature red cells which continue to be more or less actively metabolic after entering the circulating blood. The disappearance of methaemoglobin after death is presumably accelerated under these circumstances which may also operate compensatorily by way of limiting to some extent the conversion of haemoglobin during life.

(b) Apart from the presence of methaemoglobin, the blood of most of the blue rats was distinctly abnormal in other respects, more especially in the sense that more or less anaemia was present with some increase of blood volume. Neither the methaemoglobinaemia nor these other changes are constant in infected rats: the blood may be brown

<sup>1</sup> C. G. Douglas, *Journal of Physiology*, Vol. xxxix. (1910) p. 453.

<sup>2</sup> *Münchener med. Woch.* Vol. LV. (1908) p. 1817; Schmiedeberg's *Archiv*, Vol. LX. (1909) p. 298. See also M. Onaka, *Zeitschr. f. physiol. Chemie*, Vol. LXXI. (1911) p. 193, who attributes the chief action to the platelets.

but otherwise not abnormal (*e.g.* rat 1, Table II) or the blood pigment may be normal in quality but very deficient in quantity (*e.g.* rats 12 and 13, Table III). Infection may indeed be present with the usual pathological changes in the liver and spleen without any definite abnormality in the red corpuscles (*e.g.* rats 2, 3 and 4, Table III).

In examining the blood, samples for the estimation of the haemoglobin percentage and for the enumeration of red cells were obtained from the heart under chloroform anaesthesia. All the haemoglobin was then collected by washing out the circulation with citrated saline, the washings laked, mixed and titrated against standardised dilutions of rat or human blood. In titrating brown blood it is convenient to reduce both the sample and the standard with hydrosulphite immediately before titration and work with reduced haemoglobin, or, if the tint be preferred, saturate with carbon monoxide as well and titrate as CO-haemoglobin.

The distribution of the various values for normal rats has been described before for a small number of animals<sup>1</sup> and has been recently dealt with at length by Chisolm<sup>2</sup> on the basis of an extended series of measurements. His results as far as they are necessary for comparison with the present series of animals are as follows:

TABLE I. *Blood data for normal rats between 50 and 150 grammes body weight.*

	Average	Range of variation*	
		95 per cent. of normal rats are within the limits	All normal rats are within the limits
Total haemoglobin per kilo body weight expressed as cub. cent. of oxygen capacity	10·0	8·2—11·7	7·3—12·6
Blood volume per kilo body weight	63	49—76	43—83
Haemoglobin per cent. on human scale	88	71—104	63—113
Red cells in millions per cub. mill.	8·8	6·8—10·8	5·8—11·8

\* The two standards of normality are defined by the means  $\pm$  twice and three times the standard deviations respectively. These limits naturally apply only to individuals, not to the means of series.

For the purpose of calculating the colour index, the normal figures have been taken as nine million red cells and 88 per cent. haemoglobin, and in the tables the indices are given on this rat scale: on the human scale the normal colour index of the rat is 0·5. Stained blood films were also examined. In normal rats, especially young animals, a slight

<sup>1</sup> *British Medical Journal*, 5 November, 1910.

<sup>2</sup> *Quart. Journ. Exper. Physiol.* Vol. iv. (1911) p. 208.

degree of polychromasia is often present, but much polychromasia or the presence of nucleated red cells indicates active blood formation; from this, in the absence of any excess of haemoglobin, we may assume considerable blood destruction.

The quantities of haemoglobin and blood are usually expressed in terms of body weight. In sick animals however the weight may be abnormal owing to partial starvation, oedema or the like. To exclude this source of fallacy, it is desirable to have some independent criterion of bigness, the most convenient being the body length. The relation between body weight and body length in normal rats, according to Chisolm's observations, is given in Fig. 1, and the length of the

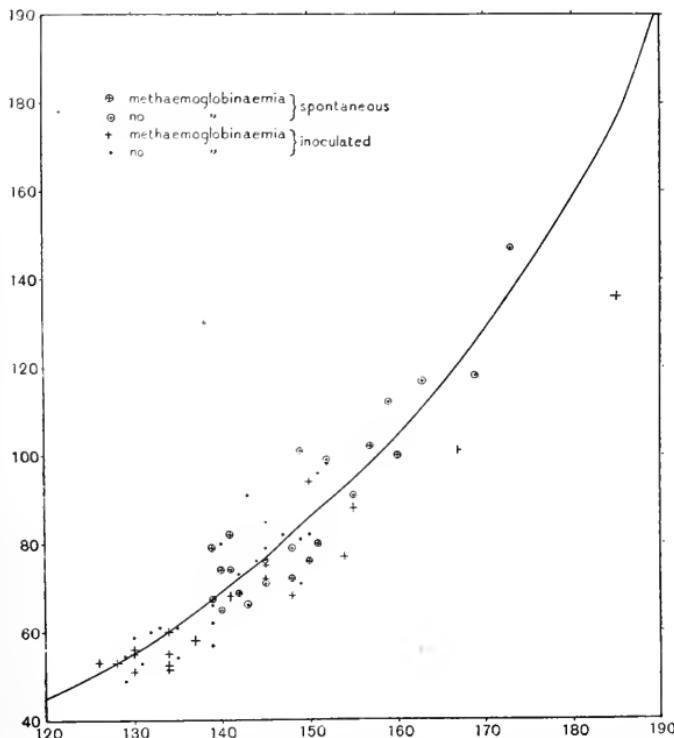


Fig. 1. Relation between body weight and body length. The normal relation is given by the continuous line. There is a tendency for the infected rats to be too light for their body length.

individual animals is shown in the tables. After giving due weight to the consideration that an animal of abnormal body weight will, in adjusting its blood to pathological circumstances, probably take some note of the actual mass of tissue subsisting at the moment, it appears likely that it is on the whole better to refer the blood data to body length as well as body weight; the quantities of haemoglobin and blood in the present series of animals calculated on this basis also are therefore given in Tables VI and VII below.

TABLE II. *Blood data for spontaneous cases of methaemoglobinæmia. Liver necrosis present and spleen cultures positive in all cases.*

Number	Sex	Body weight (grammes)	Body length (mm.)	Haemoglobin (per cent.)	Total oxygen capacity per kilo body weight* (c.c.)	Blood volume per kilo body weight (c.c.)	Polychromasia	Nucleated red cells	Colour index
1	M	102	157	71	9·9	77	+	0	1·2
2	F	67	139	63	8·5	73	+	0	1·0
3	M	79	139	59	8·2	75	+++	0	—
4	F	69	142	58	7·0	66	0	0	1·1
5	M	76	150	57	11·3	107	++	0	—
6	M	80	151	55	7·9	78	+++	+++	—
7	F	100	160	49	7·6	84	+++	0	—
8	F	72	148	48	7·8	88	+	0	—
9	M	81	141	25	4·2	91	++	+++	—
10	M	74	140	12	3·4	158	lost	—	—

\* In these figures the methaemoglobin is reckoned as if it had the oxygen capacity of normal haemoglobin.

Table II shows that most of the blue rats were also anaemic in the sense that the percentage of haemoglobin was too low; many of them also had too little total haemoglobin, and five of them had very large blood volumes. Rats 9 and 10 show very profound degrees of anaemia; the latter animal had one-third the normal amount of haemoglobin and  $2\frac{1}{2}$  times the proper quantity of blood. In no case is there any evidence of any compensatory production of normal haemoglobin to replace that which was put out of action by being converted into methaemoglobin.

In the infected rats which did not have brown blood (Table III) the changes are less pronounced. There is the same general tendency for the haemoglobin percentage to be low, the total haemoglobin to be low and the blood volume to be high, but only two (12 and 13) were severely anaemic.

(2) *Changes in other organs.*

The most marked changes are seen in the spleen and liver. The spleen is much enlarged, firm, rather pale and mottled over with white or pale yellow flecks. Histologically the pale flecks are found to be patches of necrosis; elsewhere the pulp is stuffed with blood, leucocytes and proliferating endothelial cells: in some cases, especially immediately round the necrotic areas, plasma cells are abundant; a good many are sometimes present in spleens which are apparently normal obtained from uninfected rats. The liver is rather pale and

TABLE III. *Blood data for spontaneous infected rats which did not have methaemoglobinæmia when examined. Liver necrosis present and spleen cultures positive in all cases.*

Number	Sex	Body weight (grammes)	Body length (mm.)	Hæmoglobin (per cent.)	Total oxygen capacity per kilo body weight (c.c.)	Blood volume per kilo body weight (c.c.)	Polychromasia	Nucleated red cells	Colour index
1	M	147	173	103	9·2	48	0	0	1·1
2	F	65	140	80	10·4	70	0	0	0·7
3	M	101	149	78	10·2	71	+	0	—
4	F	91	155	70	9·6	74	+	0	—
5	M	118	169	70	10·7	83	+	0	1·0
6	M	76	145	67	7·7	62	—	—	—
7	F	79	148	66	9·3	76	+	0	—
8	M	117	163	65	7·4	61	+	+	1·2
9	F	63	143	60	8·7	78	+	0	0·8
10	M	74	141	58	7·6	71	++	0	—
11	F	71	145	56	8·5	81	+	0	0·8
12	M	99	152	47	6·0	69	++	++	—
13	M	112	159	42	5·1	66	++	0	—

sometimes definitely mottled on close examination but the white flecks are never so large as in the spleen. Histologically there are abundant focal necroses. There is nothing characteristic in these lesions; they are exactly the same as those described by Ledingham<sup>1</sup> in rats infected with plague, and his description will stand as a more ample account of the changes found in the animals, blue or not, infected with Gaertner. It is possible, however, as will be seen below, that the

<sup>1</sup> *Journal of Hygiene*, Vol. vii. (1907) p. 359. I noticed the same lesions in the acute Gaertner rat epidemic which occurred here in 1908.

liver necrosis has some connection with the methaemoglobinaemia; its presence denotes at any rate a definite and effective infection as compared with an invasion of the body with bacilli which gives rise only to something akin to the "carrier" state.

(3) *Distribution of the bacilli.*

In blue rats there is always a septicaemia. Exact measurements were not made, but one drop of blood (about 0·02 c.c.) spread over an agar slope gave a continuous growth in all cases. In infected rats which were not blue, no septicaemia was generally found unless the animals were obviously ill. Cultures were usually made from the spleen whence the bacillus is readily obtained pure. Cultures from intestinal contents and scrapings of the walls of the bowels sometimes gave pure cultures but were more often negative as regards Gaertuer's bacillus<sup>1</sup>. Faeces gave the same results; the animals nearly always had more or less profuse diarrhoea.

(4) *Results of inoculation with the rat bacillus<sup>2</sup>.*

In order to reproduce the features of the natural disease by artificial inoculation, it is necessary to use appropriate doses of bacilli. If rats are given large doses (*e.g.* 1 c.c. 20 hours' broth culture intraperitoneally) of a recently isolated strain, they die within the next 48 hours and their blood is very seldom, and never more than a little, brown. After various trials a dose of about 0·2 c.c. of a 20 hours' broth culture given subcutaneously was found to give pretty good results and was generally used. Many experiments showed that with this dose some six or seven rats out of ten would generally develop methaemoglobinaemia to some extent. The results of two typical experiments are as follows:

- EXP. 1. June 21. 10 rats inoculated subcutaneously with strain B (from rat 5, Table II), 0·2 c.c. 20 hours' broth culture.
- ,, 23. 1 ill.
  - ,, 25. 2 found dead, 2 blue which were killed.
  - ,, 26. 3 blue, killed.
  - July 1. 1 found dead.
  - ,, 4. 2 survivors seem well; killed; spleen cultures negative, no liver necrosis.

<sup>1</sup> Non-lactose fermenters other than Gaertuer's bacillus are so abundant in rats' intestines that, using as I did McConkey lactose-bile-salt-agar plates, it would be easy to miss a specific organism: no exhaustive search was made.

<sup>2</sup> Albino rats were always used in order that the changes in the general appearance of the animal might be as obvious as possible.

EXP. 2. October 20. 19 rats inoculated subcutaneously with strain D (from rat 13, Table III), 0·2 c.c. 20 hours' broth culture.

- ,, 22. 3 blue.
- ,, 24. 1 dead, 7 blue.
- ,, 25. 4 dead, 4 blue and killed.
- ,, 28. 1 dead, 2 blue, one of which was killed.
- ,, 29. 2 blue, one of which was killed.
- ,, 31. 1 blue.

November 2. Blue one recovered, killed; 6 others seem well, killed; of these two gave positive spleen cultures, one of which also had necrosis of liver.

In the first case therefore of ten rats, three died without any proof that they had had methaemoglobinaemia, five became blue, and two survived. In the second experiment, in which the bacillus used had been sub-cultured on artificial media for five months, of 19 rats one died, 12 became blue, one blue one recovered (a very exceptional occurrence) and six survived. Of the eight survivors (out of 29 rats) which had never appeared ill in the two experiments, the bacillus was isolated from the spleen in only two, and in only one was necrosis of the liver present. There is therefore no evidence in six of these animals that the inoculation had given rise to any generalised infection though they were all infected in the sense that each one had the small abscess at the site of injection which is the almost constant result of subcutaneous inoculation.

Similar results were obtained with 15 different strains of Gaertner's bacillus isolated from rats which were blue, anaemic but not blue or apparently healthy. After some months' cultivation their efficiency showed some signs of falling off, but the change was not very definite. There can therefore be little doubt that *methaemoglobinaemia in rats can be produced and is directly caused by Gaertner infection*. To satisfy the possible criticism which is suggested by the fact already noted that Gaertner's bacillus was rather common among the experimental rats, three sets of experiments were done on 34 rats, in which the organs of blue rats were ground up with saline, filtered through a Berkefeld filter and inoculated in large and small doses. No illness or blueness resulted and no evidence of a "filter passer" was obtained. The rats were subsequently tested by subcutaneous inoculation of live broth cultures of the rat bacillus (strains D and 41); they proved to be susceptible to a normal degree.

The changes in the liver and spleen were just the same as in the naturally-infected animals. It was ascertained however that at least four or five days must elapse after inoculation before they reached the stage

usually found in natural infections. In five animals killed 13 or 14 days after inoculation, one of them having been blue and recovered and the other four having shown no signs of illness, a localised purulent myocarditis of the left ventricle was found—a lesion not noted in the spontaneous cases. With regard to the septicaemia, inoculation enabled one to ascertain that a slight septicaemia (two to five colonies from 0·02 c.c. of blood) was present when the animal first began to look blue or show any signs of general illness. Afterwards the septicaemia increased to the stage found in the naturally infected blue rats.

The blood was examined quantitatively in a number of inoculated animals. Table IV shows the results in a series of animals which had brown blood. The figures differ from those obtained from naturally infected blue rats chiefly in that the total oxygen capacity is not so much reduced and histological signs of blood regeneration were less marked and not infrequently absent. In two animals however—numbers 17 and 18—a notable degree of haemoglobin deficiency was achieved,

TABLE IV. *Blood data for cases of' methaemoglobinæmia produced by inoculation of bacillus from rats. Liver necrosis present and spleen cultures positive in all cases.*

Number	Sex	Body weight (grams.)	Body length (mm.)	Hæmoglobin (per cent.)	Total oxygen capacity per kilo body weight (c.c.)	Percentage of haemoglobin changed to methaemo- globin	Effective oxygen capacity per kilo body weight (c.c.)	Blood volume per kilo body weight (c.c.)	Nucleated red cells	Polychromasia	Colour index*	Days after inoculation
1	F	136	185	72	10·7	37	6·8	81	0	0	1·1	4
2	F	55	130	63	10·8	—	—	93	+	0	1·0	6
3	F	52	134	63	10·5	61	4·1	91	+	0	1·0	5
4	M	68	141	58	10·8	68	3·4	100	0	0	1·1	5
5	F	101	167	57	10·4	45	5·7	99	0	0	1·3	4
6	F	55	134	56	10·0	56	4·4	96	0	0	1·1	5
7	M	75	145	55	10·2	55	4·6	100	++	+	0·9	9
8	F	56	130	54	9·9	45	5·5	99	+	0	1·1	5
9	M	72	145	52	10·3	59	4·2	108	0	0	1·3	5
10	M	53	126	51	8·7	60	3·5	93	+	0	0·8	9
11	F	88	153	51	8·1	32	5·5	87	0	0	0·9	7
12	M	60	134	50	8·1	51	4·0	88	+	+	1·1	9
13	M	94	150	50	6·9	trace	6·9	74	++	0	1·2	18 (?)
14	M	52	134	43	8·3	18	6·8	105	0	0	1·0	5
15	F	58	137	42	7·6	36	4·9	98	++	+	1·1	5
16*	F	77	154	41	7·5	58	3·1	99	++	0	1·1	14 (?)
17	F	68	148	28	5·4	54	2·5	105	0	0	1·0	6
18	M	51	130	26	5·1	15	4·4	107	+	0	1·1	6

\* This rat was found naturally infected with *Trypanosoma lewisi*.

and if allowance is made for wasting and the figures calculated on the weight normal for the observed body length rather than on the observed weight at the time of death most of the rats prove to be short of haemoglobin; this is shown graphically in Fig. 2. In other respects the results closely resemble those found in spontaneous blue rats. Fifteen of the eighteen animals have a low percentage of haemoglobin outside the normal limits and sixteen have an equally abnormal high blood volume. On the average the blood volume exceeds the normal by about 50 per cent.

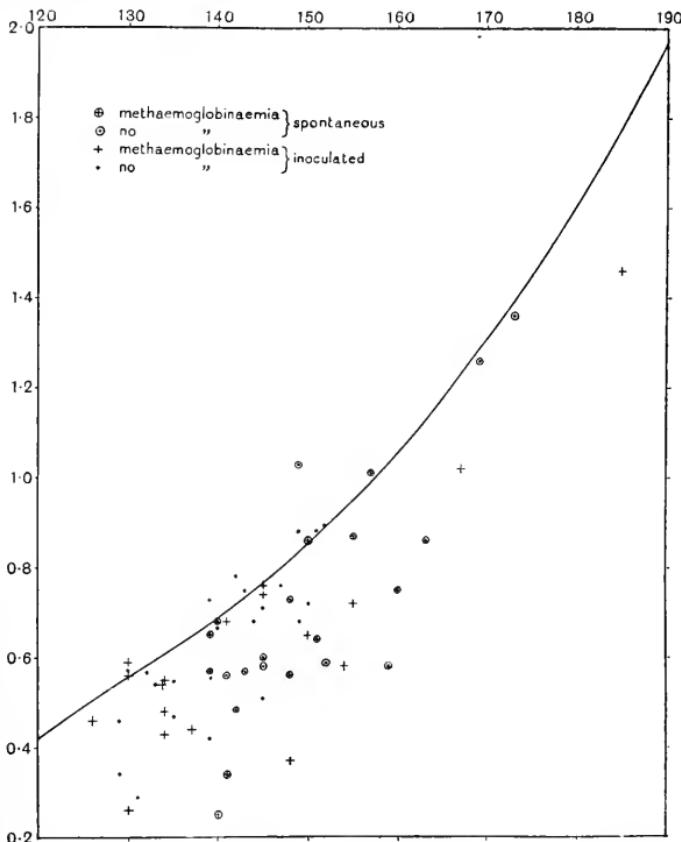


Fig. 2. Relation between body length and total oxygen capacity. The normal relation is given by the continuous line. Most of the animals are anaemic.

Information was obtained from this series of animals with regard to the proportion of haemoglobin converted into methaemoglobin. At first various colorimetric methods were tried<sup>1</sup>: they proved unsatisfactory. Good results were however obtained as follows. About 2 c.c. of blood was obtained from the heart and the oxygen capacity of the haemoglobin and the methaemoglobin together was determined, after saturation with CO and reduction, with the haemoglobinometer in the usual way. After saturation with air the oxygen capacity was directly determined by the ferricyanide method in the Barcroft-Haldane-Brodie apparatus<sup>2</sup>.

TABLE V. *Blood data for animals inoculated with the rat bacillus which did not develop methaemoglobinaemia.*

Group A. Animals showing signs of general infection when killed.

Number	Sex	Body weight (grams)	Body length (mm.)	Hæmoglobin (per cent.)	Total oxygen capacity per kilo body weight (c.c.)	Blood volume per kilo body weight (c.c.)	Polychromasia	Nucleated red cells	Colour Index	Days after Inoculation
1	F	57	139	77	10·0	70	+	0	1·0	13
2	M	82	150	76	8·8	76	0	0	1·0	13
3	M	66	139	73	11·0	81	0	0	1·0	13
4*	F	73	142	64	10·7	91	++	+	1·0	13
5	F	76	144	60	8·9	81	+	0	1·0	13
6	F	85	145	60	8·4	75	0	0	1·0	12
7	M	61	135	56	9·0	87	++	+	0·9	10
8	M	49	129	53	9·5	96	++	+	1·0	12
9	F	54	135	46	8·8	103	+	+	0·9	10
10	F	54	129	40	6·3	85	++	+	1·3	9
11	M	79	145	37	6·4	95	+++	+	1·1	12

Group B. Animals showing no signs of infection when killed.

1	F	71	149	97	12·4	69	0	0	1·0	16
2	M	91	143	82	8·2	54	+	0	1·1	18
3	M	96	151	77	9·1	64	++	0	1·0	14
4	M	82	147	75	9·2	67	0	0	1·0	12
5	M	98	152	72	9·0	68	0	0	1·0	13
6	M	81	149	69	8·4	66	0	0	0·9	14
7†	F	60	132	61	9·6	85	+	+	1·0	9

\* This rat had well-marked methaemoglobinaemia but had recovered.

† This rat was found naturally infected with *Trypanosoma lewisi*.

<sup>1</sup> See J. S. Haldane, R. H. Makgill and A. E. Mavrogordato, *Journal of Physiology*, Vol. xxI. (1897) p. 169.

<sup>2</sup> *Journal of Physiology*, Vol. xxviii. (1902) p. 232. The present much improved form may be obtained from F. P. Rittershaus, 47 Gray's Inn Road, W.C.

The whole procedure occupies only a few minutes and the results seem quite satisfactory if care is taken to obtain the maximum reading before oxygen begins to be absorbed in appreciable quantities: this is especially liable to happen if the blood contains immature red cells<sup>1</sup>. The difference between the two determinations gives the amount of methaemoglobin. For example, the haemoglobinometer readings were 44 and 45 per cent., mean 44·5 per cent., giving an oxygen capacity of  $\frac{44\cdot5 \times 18\cdot5}{100} = 8\cdot23$  c.c. per cent. The same blood gave by direct experiment an actual oxygen capacity of 3·29 per cent. The methaemoglobin was therefore  $8\cdot23 - 3\cdot29 = 4\cdot94$  or 60 per cent. of the whole. The data given in Table IV were obtained in this way. From there it appears that in an average case about half the haemoglobin is converted; in no case was a conversion of more than two-thirds observed.

A number of the rats inoculated with the rat bacillus did not develop obvious methaemoglobinaemia<sup>2</sup>. On some of these blood examinations were made. The results are shown in Table V which includes only the survivors from batches of inoculated rats, some of each lot having developed methaemoglobinaemia. The first eleven animals were found post-mortem to have definite signs of general infection—liver necrosis, myocarditis or the characteristic spleen containing the bacillus. These show much the same blood changes as were found in the blue animals but to a less marked degree; the severe anaemias observed in what may be regarded as the corresponding group of naturally infected animals (see Table III) were not reproduced. In the remaining rats there was a small abscess at the site of inoculation but no signs of general infection, nor was the organism isolated from the spleen. The blood data show only a somewhat doubtful tendency towards slight anaemia.

In considering the significance of these various changes in the blood it is necessary to separate, as far as may be, those which may be due to the presence of methaemoglobin from those due to the infection. The diminution of haemoglobin percentage, the increase in blood volume, the diminished total oxygen capacity and the histological signs of

<sup>1</sup> C. G. Donglas, *Journal of Physiology*, Vol. xxxix. (1910) p. 458.

<sup>2</sup> That is, did not appear blue or anaemic on careful comparison with healthy white rats. It did not seem necessary to obtain actual samples of blood in all cases, and indeed it is hardly fair to the experiment to do so daily. The presence of small degrees of methaemoglobinaemia cannot therefore be positively affirmed to have been absent in all cases though, in the rats which were examined, no methaemoglobin was ever found unless a moderate suspicion of its presence had been aroused by the general appearance of the animal.

regeneration in the blood are probably due to the infection alone. Chisolm<sup>1</sup> has pointed out that these changes may occur also in rats bearing transplanted sarcomata, as well as in those infected with Gaertner's bacillus, *Bacillus suipestifer* (*B. aertryck*), or rat scabies. In some cases the only change is a diminution of haemoglobin percentage with an increase of blood volume, in other words an anaemic plethora rather than a true anaemia. As has been shown, the most constant change in

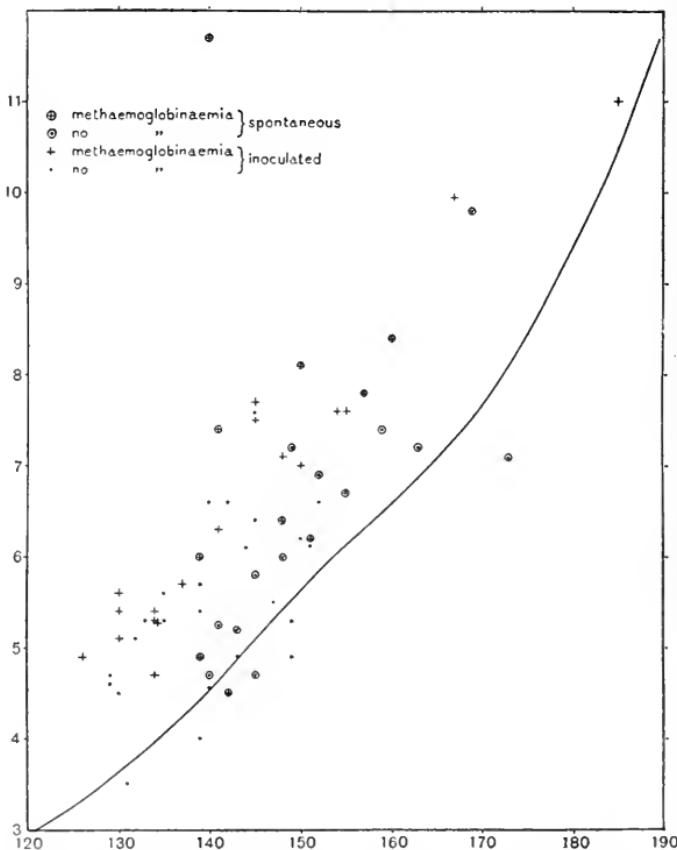


Fig. 3. Relation between body length and blood volume. The normal relation is given by the continuous line. Most of the animals have too much blood.

<sup>1</sup> *Journal of Pathology*, Vol. xv. (1911) p. 358; Vol. xvi. (1911) p. 152.

the Gaertner-infected rats has been a tendency towards the same condition, and this therefore is probably attributable to general illness of almost any kind. Table VIII gives the blood changes found in a few animals inoculated with *B. suispester*, showing that much the same condition is produced as by inoculation with Gaertner's bacillus.

TABLE VI. *Showing the total haemoglobin in terms of the normal values for rats of the given body lengths: normal = 100\**.

Naturally infected rats		Inoculated rats	
blue (Table II)	not blue (Table III)	blue (Table IV)	not blne (Table V)
Number	Number	Number	Number
1	103	1	83
2	84	2	106
3	96	3	91
4	67	4	95
5	100	5	85
6	72	6	92
7	73	7	99
8	68	8	101
9	48	9	96
10	37	10	90
		11	79
		12	81
		13	75
		14	72
		15	68
		16	62
		17	45
		18	48
		A 1	84
		2	83
		3	107
		4	109
		5	89
		6	92
		7	89
		8	86
		9	76
		10	63
		11	66
		12	105
		13	101
		14	100
		15	94
		16	100
		17	81
		18	100

\* The limits of normality for individuals are (Table I) 82-117 and 73-126.

Owing to the presence of methaemoglobin any anaemia is in effect exaggerated and there may be an extreme defect in the actual oxygen-carrying power of the blood. Thus in rat 17 of Table IV, the blood per unit volume had an actual and effective oxygen capacity of only one-quarter of the normal, and in rats 9 and 10 of Table II this may be presumed to have been reduced to about one-fifth. Rats with the full normal quantum of red blood corpuscles also become effectively anaemic; thus in Table IV there are seven animals (1, 3, 4, 5, 6, 7 and 9) with more than 10 c.c. total oxygen capacity per kilo, the mean being 10·4 c.c. On the average 54 per cent. of haemoglobin is methaemoglobin, and the effective oxygen capacity varies from 3·4 c.c.

to 6·8 c.c. per kilo, mean 4·7 c.c. Under these circumstances it might be expected that some over-production of haemoglobin would occur in an attempt to compensate for that put out of action as happens in animals in which a proportion of the haemoglobin is rendered useless

TABLE VII. *Showing the blood volumes in terms of the normal values for rats of the given body lengths: normal = 100\**.

Naturally infected rats		Inoculated rats	
blue (Table II)	not blue (Table III)	blue (Table IV)	not blue (Table V)
Number	Number	Number	Number
1	126	1	99
2	114	2	148
3	139	3	125
4	100	4	141
5	150	5	131
6	112	6	140
7	128	7	154
8	121	8	161
9	165	9	160
10	269	10	153
	11	11	133
	12	12	139
	13	116	B 1
		13	129
		14	143
		15	138
		16	129
		17	136
		18	157
			10
			94
			115
			125
			146
			128
			132
			135
			139
			143
			135
			154
			93
			105
			111
			107
			119
			100
			141

\* The limits of normality for individuals (Table I) are 78–121 and 68–132.

TABLE VIII. *Blood data for animals inoculated with Bacillus suispestifer.*

Number	Sex	Body weight (grams.)	Body length (mm.)	Haemoglobin (per cent.)	Total oxygen capacity per kilo body weight (c.c.)	Blood volume per kilo body weight (c.c.)	Polyhronasias	Nucleated red cells	Colour index	Days after inoculation
1	F	59	130	68	9·6	76	0	0	0·8	12
2	F	61	133	55	8·9	87	+	0	0·9	8
3	M	80	140	55	8·4	82	++	+	1·1	12
4	F	53	131	45	5·5	66	++	+	1·2	8
5	F	62	139	40	6·8	92	+	0	1·1	8

by chronic CO-poisoning<sup>1</sup>. There is however no evidence that this occurs except that histological signs of active blood formation may occasionally (rat 5, Table II, rat 7, Table IV, rat 4, Table V) be present in animals which already have, for their weight and length, fully the normal amount of haemoglobin—an observation which becomes perhaps less significant when it is noted that these animals also had blood volumes much too big, and bigger than similar rats in which no signs of regeneration were present. The time during which a substantial degree of methaemoglobinaemia prevails might appear too short for compensatory production of red cells: this however is not the case since experiments show that after haemorrhage<sup>2</sup> or under the influence of diminished atmospheric pressure a rat may generate an amount of haemoglobin equal to half its normal quantum within a week. The failure of compensation may therefore be attributed to the sickness of the animal. In general we may note that any production of fresh haemoglobin would not necessarily be effective since there are grounds for supposing that the amount converted into methaemoglobin is not determined by the absolute activity of the methaemoglobin-producing mechanism but in a proportion which is a product of the balance between this mechanism and the reducing substances present in the blood.

#### (5) *The occurrence of the bacillus in rats.*

The same bacillus was invariably obtained from naturally infected rats with methaemoglobinaemia. Of these during nine months ten were identified out of rather more than 1000 rats which were used in the laboratory during that time. No doubt more occurred without being noticed, though the absence of any obviously excessive mortality among the stock rats excludes any considerable prevalence of methaemoglobinaemia at any time. The numerical frequency of infected rats which did not show methaemoglobinaemia cannot be definitely stated, since at first only minute fragments of splenic tissue were cultivated. Later on the whole spleen was incubated in dulcite bile-salt broth<sup>3</sup>, and if acid and gas developed sub-cultured in the same medium before

<sup>1</sup> G. G. Nasmith and D. A. L. Graham, *Journal of Physiology*, Vol. xxxv. (1906) p. 32. Polycythaemia is described in some human cases of chronic methaemoglobinaemia from poisoning with e.g. acetanilide, but the total haemoglobin does not appear to have been determined.

<sup>2</sup> *Journal of Pathology*, Vol. xvi. (1911) p. 269.

<sup>3</sup> On the advantages of preliminary cultivation in dulcite media for the isolation of organisms of this group, see Boycott, *Journal of Hygiene*, Vol. vi. (1906) p. 35.

plating. In this way it may more fairly be assumed that any small number of bacilli in the spleen would be found; using only such bits of splenic pulp as may be dug out with a platinum needle, it is doubtful whether very much weight ought to be attached to negative results. Working in this way, 50 consecutive apparently normal rats were examined, most of which had been killed for other purposes. The bacillus was isolated from the spleen in eight and from the intestines once. Of these nine rats, liver necrosis was present in five and absent in four. In another series of 27 rats which died after inoculation with pneumococci, six were found infected by spleen culture, in five of which liver necrosis was present. Roughly speaking therefore about 20 per cent. of the animals seem to have harboured the bacillus.

During the course of the experiments, cultures were made from ten wild rats (*Mus decumanus* sp. *norvegicus*) caught in the animal houses and the laboratory: Gaertner's bacillus was isolated from the spleen of one of them.

#### (6) *Modes of infection.*

Infection is conveyed by feeding but not apparently with any great certainty. Of four rats fed with half a 20 hours' agar culture, one became blue; of six rats fed with two old agar cultures and 20 c.c. of four days old broth culture, two became blue; and of four fed on the corpse of a blue rat, none gave positive results.

Infection also results by living together<sup>1</sup>. Thus six rats, which had been under observation for eight days, had added to their cage three rats blue from inoculation; these died on the first, second and eighth days respectively, their bodies being removed uneaten. Of the normal rats two developed methaemoglobinaemia and died on the eleventh day, and another on the sixteenth day: the other three survived.

If contact and feeding are combined, infection may spread more widely. Thus, in one experiment, to a cage of nine normal rats were added, over a space of 13 days, seven rats which had developed methaemoglobinaemia in sequence to inoculation; their bodies were all more or less eaten after death. One normal rat died on the eighth day, but the spleen and liver were normal and the spleen culture negative. On the thirteenth day the other eight were removed to a clean cage, and

<sup>1</sup> As already noted, the bacillus is not readily obtained from the bowels. Considering however the facts that the evacuations are very often blood-stained and that the organism is present in the blood in large numbers, this is probably due to defective technique.

during the next four days five of them developed methaemoglobinaemia and died. As in the previous experiment, there was some lag in the development of infection.

Fleas were never found on the stock rats. Infection was however to some extent present in the wild rats living in the neighbourhood and these harbour both *Ceratophyllus fasciatus* and *Pulex cheopis* in considerable numbers<sup>1</sup>. Several experiments with both parasites were done<sup>2</sup> to see whether infection would spread from rat to rat by contiguity without contact in the presence of abundant fleas and whether infection could be transferred by transferring fleas which had sucked the blood of blue rats. They were all negative. By cultivating fleas which have fed on blue rats, the bacillus may be recovered pretty easily on the first and second day, but on the fifth day and later the results were uniformly negative.

#### (7) *The identity of the causative organism*<sup>3</sup>.

The organism isolated corresponds culturally with *B. enteritidis* of Gaertner. It is an actively motile, Gram-negative bacillus growing freely on ordinary media at room temperature and in the incubator and, though rather less luxuriantly, anaerobically: no indol: no liquefaction of gelatin: litmus milk acid for the first two or three days, later becoming markedly alkaline. Grown in broth, it ferments and produces gas with dextrose, laevulose, galactose, maltose, dulcite, mannite, sorbite and arabinose, but gives no change with lactose, cane-sugar, glycerin, dextrin, raffinose, inulin, adonite, amygdalin or erythrite. A good deal of confusion arose at first from the fact that strains freshly isolated from rats generally produced acid only, and never more than a bubble or two of gas, when grown in maltose broth in the usual way with Durham's tubes. Bainbridge has noted that laboratory cultures of

<sup>1</sup> *Guy's Hospital Gazette*, Vol. xxv. (1911) pp. 73, 318.

<sup>2</sup> These experiments were done at the time of year when the original epidemic occurred; this may be an important point as has been shown in the case of plague transmission (*Journal of Hygiene*, Vol. viii. (1908) p. 279).

<sup>3</sup> I am very much indebted to Dr F. A. Bainbridge who furnished me with the standard cultures of Gaertner's bacillus, *B. aertryck*, paratyphoid A, paratyphoid B, and *B. suispestifer*, which he used in his enquiry into this group of organisms (*Journal of Pathology*, Vol. xiii. (1909) p. 443), and was also good enough to give me agglutinating rabbit sera prepared with them. See also on the differentiation of these Gaertner strains H. R. Dean, *Proc. Roy. Soc. Med.* 1911, Vol. iv. Path. Sect. p. 251; G. Sobernheim and E. Seligmann, *Zeitschr. f. Immunittsrf.* Vol. vii. (1910) p. 342.

Gaertner's bacillus and the allied organisms produce less gas from maltose than from the other fermentable sugars, and in my own strains definite, though always small, gas production appeared after several sub-cultures outside the body. In isodulcite (rhamnose) the standard Gaertner produced acid and a little gas in 24 hours, whereas the rat bacilli caused no change for a week and then only acid appeared.

Culturally therefore the organism belongs to one of the three groups, (1) Gaertner's bacillus, (2) *B. aertryck* and *B. suispestifer* or (3) paratyphoid B. That the rat bacillus is a true Gaertner is shown by agglutination and absorption tests.

TABLE IX. *Agglutination with standard Gaertner serum (Bainbridge).*

	1/500	1/1000	1/2000	1/5000	1/10,000
Standard Gaertner	+++	+++	+++	++	0
Rat bacillus A	+++	+++	+++	++	0
" D	+++	+++	+++	++	0
" M	+++	+++	+++	++	+
" 16	+++	+++	+++	+	0
" 9	+++	+++	+++	+	0
Suapestifer	0	0			
Paratyphoid B	0	0			
Suapestifer E	0				
Gaertner 2	+++	+++	+++	++	+
Suapestifer 4	0	0			
Gaertner R	+++	+++	+++	++	+
Suapestifer M	0	0			

The Gaertner serum was then absorbed with excess of standard Gaertner, rat bacillus M, 16 and 9, suapestifer and paratyphoid B, the growth off two 48 hours' agar slopes being mixed with 0·2 c.c. serum diluted  $\frac{1}{50}$  for two hours at 37°. The concentration of serum in the test was therefore between  $\frac{1}{100}$  and  $\frac{1}{200}$ .

TABLE X.

Absorbed with:-	Standard Gaertner	M	16	9	Suapestifer	Paratyphoid B
Standard Gaertner	0	0	0	0	+++	+++
Rat bacillus M	0	0	0	0	+++	+++
" 16	0	0	0	0	+++	+++
" 9	0	0	0	0	+++	+++

With Aertryck (*suapestifer*) agglutinating serum, the homologous organisms reacted  $\frac{1}{2000}$  to  $\frac{1}{5000}$ ; rat bacillus A, D and M gave no agglutination at  $\frac{1}{200}$ . Further experiments were made with the sera of

rabbits repeatedly inoculated with dead, and later living, cultures of rat bacillus D and M. Both sera gave the same result: those for strain D were as follows:

TABLE XI. *Agglutination with rat bacillus serum, strain D.*

	1/20	1/100	1,500	1,1000	1/2000	1/5000	1/10,000	1/20,000
Rat bacillus D	+++	+++	+++	+++	+++	++	++	+
" 19	+++	+++	+++	+++	+++	++	++	+
" B	+++	+++	+++	+++	++	++	++	+
" Y	+++	+++	+++	+++	+++	+++	+++	++
Standard Gaertner	+++	++	+++	+++	+++	+++	+++	++
Suipestifer	+++	0	0	—	—	—	—	—
Paratyphoid B	++	++	+	0	—	—	—	—

After absorption (diluted  $\frac{1}{500}$ ) the results were:

TABLE XII.

Absorbed with :-	Paratyphoid B	Suipestifer	D	B	Gaertner
Rat bacillus D	++	++	0	0	0
" 19	++	++	0	0	0
" B	++	++	0	0	0
" Y	++	++	0	0	0
Standard Gaertner	++	++	0	0	0
Suipestifer	0	0	0	0	0
Paratyphoid B	0	0	0	0	0

These results show clearly therefore that *the rat bacillus is Gaertner's bacillus* in the sense that it is indistinguishable from Bainbridge's standard strain of Gaertner, i.e. from the original strain isolated by Gaertner. They also demonstrate that the various strains of the rat bacillus isolated at different times are identical. Strains A, B, 16 and 19 were isolated from rats with spontaneous methaemoglobinaemia on 13th May, 18th May, 30th November and 8th December respectively; D, M, Y and 9 from rats without methaemoglobinaemia, and in the case of 9 without signs of illness, on 24th May, 29th June, 2nd November, and 22nd November respectively.

#### (8) *Inoculation with other strains of Gaertner's bacillus.*

Experiments were made with five other strains of Gaertner's bacillus, all of which had been identified by agglutination and absorption as well as by cultural tests.

Of strains isolated from rats I had two. One of these (Gaertner R, Table IX) had been obtained from the acute epidemic here in 1908.

I am indebted to Dr Eyre for its preservation. With doses of 0·2 c.c. 20 hours' broth culture subcutaneously, of ten rats, five died without developing methaemoglobinaemia (two on the first day, one on the second, two on the fourth) and one was blue and died on the fifth day. For the other rat strain I have to thank Dr Bainbridge who isolated it recently (1911) from another acute epidemic. With the same dose, of ten rats, four showed well-marked methaemoglobinaemia, four died and two survived.

I have also to thank Dr Bainbridge and Dr O'Brien for a Gaertner culture recently isolated from a natural epidemic among guinea-pigs<sup>1</sup>. Ten rats received 0·5 c.c. 20 hours' broth subcutaneously; they all had exceptionally large lesions at the site of inoculation but only one became blue and died, the other nine showing no signs of illness.

Of old laboratory strains, incomplete experiments have been made with two. The original standard strain of Gaertner gave negative results, but after six quick passages through the peritoneal cavity of rats, two animals out of ten developed methaemoglobinaemia. Another old strain (Gaertner 2, Table IX) from a human source given me by Dr Eyre was also negative at first, but after passage gave positive results in six out of eight rats.

In the present instance there is of course more than the usual possibility that the organism recovered from the passage animals was not the same as that put into them. Assuming however that no change occurred, all five strains of Gaertner, from rats, guinea-pigs and men, produced methaemoglobinaemia under appropriate circumstances.

#### (9) *Vaccination experiments.*

Further evidence with regard to the relationship of the rat bacillus and Gaertner's bacillus to the disease was obtained by vaccination experiments.

(a) Ten rats received subcutaneously 0·3 c.c. 20 hours' broth culture of the rat bacillus (strain D) heated to 60° C. for 1½ hours: fifteen days later they were inoculated with 0·2 c.c. live culture (strain D). Two died, seven went blue and one survived. Of 19 controls six died, seven went blue and six survived.

(b) 17 rats had 0·2 c.c. subcutaneously of a 20 hours' broth culture of the rat bacillus (strain D) heated to 55° C. for 1½ hours, and twelve

<sup>1</sup> *Journal of Pathology*, Vol. xvi. (1911) p. 145.

days later a second dose of 0·5 c.c. of a similar preparation. Twelve days afterwards, eight of them were inoculated with 0·5 c.c. live culture (strain 41) subcutaneously: five went blue, three survived; of ten controls all died in seven days, nine being found blue before death. The other nine vaccinated rats were exposed to contact with blue rats (strain D): one died, four went blue and four survived.

(c) 17 rats were inoculated subcutaneously with 0·2 c.c. 20 hours' broth culture of the original Gaertner strain which was relatively avirulent: three died. The remaining 14 animals, 12 days later, received 0·5 c.c. 20 hours' broth culture of rat bacillus (strain 41) and two died; after further 11 days the 12 survivors had 1 c.c. subcutaneously and all survived.

Vaccination with dead cultures seemed therefore to confer no protection. With a live culture however 12 of 17 rats resisted a dose which killed all of ten controls.

#### (10) *Experiments with animals other than rats.*

A number of experiments were made with rabbits, guinea-pigs and mice, but in no case was methaemoglobinæmia produced by inoculation with any strain of Gaertner's bacillus. The inoculated animals either presented no signs of illness or developed a septicaæmia and died; in no case was any necrosis of liver found similar to that which appears to be constant in infected rats. Particular attention was paid to mice, and the dose given carefully adjusted so that they died in about five days; no trace of brownness could however be found in the blood of 40 animals. Guinea-pigs inoculated with the Gaertner strain derived from a guinea-pig epidemic did not show methaemoglobinæmia.

#### (11) *Experiments on rats with other organisms.*

As regards other members of the Gaertner group, rats were inoculated with the standard strains of paratyphoid A, paratyphoid B, *B. aertryck* and *B. suis* as well as with three other laboratory strains which proved to be *suipotifer* (*suipotifer* E, 4 and M, Table IX). One rat inoculated with paratyphoid A developed a slight degree of methaemoglobinæmia and from the spleen paratyphoid A alone was recovered; the possibility of this case being a natural infection with Gaertner's bacillus cannot be altogether excluded. Otherwise the results were all negative. The organisms are all more or less pathogenic for rats, cause

a large local lesion at the site of inoculation and in a proportion of cases liver necrosis also. Experiments after passage through rats have not yet been made.

Many inoculations were done with the pneumococcus, three strains recently isolated from human beings by inoculation of rabbits being employed. This organism was used because rats can be killed by it with a sub-acute illness and because it is *in vitro* exceptionally active in producing methaemoglobin from blood. No cases of methaemoglobinaemia arose in the inoculated rats and, in the absence of Gaertner infection, liver necrosis was not found. As it happened, among the rats dying of pneumococcus infection there were several which proved on spleen culture to be examples of spontaneous Gaertner infection.

#### (12) *Pathogenesis of methaemoglobinaemia.*

The nature of methaemoglobin and its relation to haemoglobin are unfortunately not understood. It seems however clear that in methaemoglobin and in oxyhaemoglobin the oxygen is joined on to the rest of the molecule in different ways, the amount of oxygen being the same in both cases. Methaemoglobin may be produced from oxyhaemoglobin by allowing sterile blood to stand, especially at body temperature, and also by a large number of oxidising agents; it is perhaps open to doubt whether the "reducing" agents (*e.g.* nitrites) which make methaemoglobin do so by actual reduction. By reducing agents methaemoglobin is immediately converted to reduced haemoglobin and to oxyhaemoglobin if free oxygen is available<sup>1</sup>.

How the methaemoglobin is produced in rats by Gaertner infection I have not been able to discover. There are however a variety of considerations which may be noted with a view to limiting the discussion of the problem.

The methaemoglobin is in the rats entirely within the corpuscles. Hence the agent which produces it may be presumed to be one which can penetrate the red corpuscles. Potassium ferricyanide for example does not cause methaemoglobinaemia when injected directly into the circulation since the corpuscles are impermeable to it; for the same reason chlorates do not produce methaemoglobinaemia in the rabbit though they do in man. It is conceivable that the corpuscles containing methaemoglobin have been so made in the bone-marrow; the rapidity

<sup>1</sup> I have been over most of the ground with rats' blood and haemoglobin without finding that they differ from those more commonly employed.

with which more than half the haemoglobin may be altered and the absence from the blood, in some cases, of any marked signs of marrow activity render this view hardly possible.

In the next place the persistence of marked methaemoglobinaemia for two, three or even four days before death shows that the substance or mechanism which produces methaemoglobin must be in continued action during that period. At all times in the circulating blood the proportion of haemoglobin converted must be the result of a balance between the methaemoglobin-forming activity and the reducing activity. All the circulating haemoglobin cannot be turned into methaemoglobin because of the reducing substances present in the blood. After death, when the supply of oxygen fails, these reducing substances quickly cause the disappearance of all the methaemoglobin. The same happens during life if methaemoglobin is formed as the result of the administration of a single dose of a suitable poison. Thus a rabbit may be very nearly killed by a dose of sodium nitrite<sup>1</sup> or of the very active phenyl-hydroxylamine<sup>2</sup>, but if it can tide over the worst period of oxygen deficiency the methaemoglobin is soon reduced and in a few hours the animal is practically well again.

It appears that the agent which produces methaemoglobin is not in the blood. If one makes mixtures of normal blood and blood from a blue rat there is no increase in the proportion of methaemoglobin in the mixture either at once or after incubating at 37° for six or eight hours with or without a stream of oxygen bubbling through.

The bacilli themselves are not direct producers of methaemoglobin. Animals inoculated with large doses of dead bacilli do not develop methaemoglobinaemia. If the bacilli are grown on blood-agar or in solutions of blood (either from rabbits or rats), methaemoglobin may be produced, but not invariably, after three or four days' incubation. The same result may however be obtained with many different organisms or even with sterile blood. In the same way neither live nor dead bacilli would produce methaemoglobin within a few hours from whole or laked blood if the mixture was constantly aerated with a stream of oxygen. Experiments with media of which rat formed the basis instead of cow were also negative, as were also those in which the organisms were grown in fresh or heated rat serum mixtures.

<sup>1</sup> Haldane, Makgill and Mavrogordato, *Journal of Physiology*, Vol. xxi. (1897) p. 171.

<sup>2</sup> Lewin, Schmiedeberg's *Archiv*, Vol. xxxv. (1895) p. 401. I am much indebted to Mr W. C. Ball who made some of this unstable substance for me: it produces nothing else but methaemoglobin in the blood of rabbits.

No extract—watery, aleoholic or chloroformic—could be obtained from blue rats which would *in vitro* produce methaemoglobin.

A series of experiments was also made in which the organs of blue rats were washed free of blood, pounded up, suspended in normal rats' blood and incubated with a stream of oxygen bubbling through. In these the liver in some experiments gave very definite results, a large proportion of the haemoglobin being converted to methaemoglobin in one hour. Controls with the livers of normal rats, with the livers of both blue and normal rats which had been killed by immersion in alcohol for several days and with muscle, kidney<sup>1</sup>, etc., also usually gave a slight production of methaemoglobin, which was absent from the tubes containing blood alone. Though there was therefore no absolute distinction between blue and normal animals, the quantitative difference seemed pretty clear.

These experiments suggest at once that there is some connection between liver necrosis and methaemoglobinaemia. It will be remembered that extensive liver necrosis was an invariable accompaniment of methaemoglobinaemia both in the naturally and experimentally infected rats, and that it was, equally with the methaemoglobinaemia, absent in mice, guinea-pigs and rabbits killed with the rat bacillus. On the other hand, but clearly with a significance not necessarily as great, liver necrosis was present in a certain number of rats infected with Gaertner's bacillus, and in some infected with other members of the Gaertner group, in which methaemoglobinaemia was not observed<sup>2</sup>. I am inclined therefore at present to attach to the liver necrosis some aetiological connection with the methaemoglobinaemia.

In their human case of idiopathic methaemoglobinaemia ("microbic cyanosis") Gibson and Douglas<sup>3</sup> report the finding of nitrates in the blood serum. It seemed therefore desirable to consider the possibility of the rat methaemoglobinaemia being due to nitrite poisoning<sup>4</sup>. The changes in the blood pigment in poisoning by nitrates and allied substances are fully described by Haldane, Makgill and Mavrogordato<sup>5</sup>; they point

<sup>1</sup> The spleen cannot be washed free of blood and no experiments were made with it.

<sup>2</sup> Dr Rowland tells me that he has not noticed methaemoglobinaemia in plague-infected rats in which liver necrosis is generally extensive.

<sup>3</sup> *Lancet*, 1906, Vol. II, p. 72.

<sup>4</sup> Cf. the suggestion, strange enough, that cholera is fatal through nitrite poisoning; see R. Emmerich, A. A. Hymans, van den Bergh and A. Grutterink in *Berliner klin. Woch.* 1909, pp. 2008, 2229; and 1910, pp. 779 and 1320; J. Choukewitch, *Jnn. Inst. Past.* Vol. xxv. (1911) p. 433.

<sup>5</sup> *Journal of Physiology*, Vol. xxi. (1897) p. 160.

out that nitrites produce NO-haemoglobin as well as methaemoglobin. The presence of the former may be detected by the fact that it persists after reduction and that therefore if a mixture of methaemoglobin and NO-haemoglobin is treated with reducing agents the continuous band of reduced haemoglobin will be intensified at two points corresponding to the position of the two prominent bands of NO-haemoglobin. Since NO-haemoglobin is pink (though not so pink as CO-haemoglobin) such a mixture will also differ in colour from a solution of methaemoglobin, both before and after reduction and saturation with carbon monoxide.

Further, if the body of an animal killed by sodium nitrite be left till putrefaction begins, the tissues are distinctly reddish; in the same way the ultimate product of reducing agents is NO-haemoglobin if the blood contains an excess of nitrites. In none of these points did the blood of the blue rats resemble that of nitrite poisoning. It should however be pointed out that the presence of NO-haemoglobin is at least not easy to detect if such a minimal dose of nitrite is administered to a rat that about two-thirds of the haemoglobin remains unchanged. My experiments indicated however that the difference could be detected with fair certainty (especially by putting the animal in the incubator over-night) if as much as half the haemoglobin was altered. As already shown (Table IV), in many blue rats more than 50 per cent. of the blood pigment is methaemoglobin.

The quantity of nitrite required to keep rats blue for two days would also be considerable. If given by the mouth, about 0.5 grammes is wanted about every 8 hours to produce definite lividity in a rat of about 100 grammes. More definite experiments in which sodium nitrite was injected intraperitoneally were made on a series of rats: the animals all received the same dose and were killed at intervals afterwards, the proportion of altered haemoglobin in their heart blood being estimated as in the blue Gaertner rats (see above, p. 456). Table XIII

TABLE XIII.

Dose of sodium nitrite per kilo	Killed, hours after	Proportion of unaltered haemoglobin in heart blood	Dose of sodium nitrite per kilo	Killed, hours after	Proportion of unaltered haemoglobin in heart blood
0.02 gms.	$\frac{1}{2}$	74 %	0.05 gms.	$\frac{1}{2}$	44 %
"	1	76	"	1	58
"	2	74	"	2	44
,	3	92	"	$2\frac{1}{2}$	54
				3	67
				4	84

shows the results from which it appears that a rat of 100 grammes would require about ten doses of 0·005 gms. sodium nitrite to keep its blood approximately half converted for twenty-four hours. It is not easy to imagine where such a quantity of nitrite as this could come from. The rat bacillus does not appear to produce nitrites and it actually destroys traces of nitrites added to, or present in, ordinary broth or peptone water<sup>1</sup>. The nitrite being used up in producing methaemoglobin, it is perhaps not conclusive evidence that no clear proof of the presence of nitrites in the blood or organs of blue rats could be obtained. Serum and plasma were tested directly; the other organs and whole rats by mincing them up, acidifying with phosphoric acid and distilling, the distillate being tested with  $\alpha$ -naphthylamine and sulphanilic acid. On a few occasions a slight reaction was obtained by this very delicate reagent, but equally positive results were given by normal rats treated in the same way. I conclude that this infective methaemoglobinaemia in rats is not directly due to nitrite poisoning.

#### SUMMARY.

1. A spontaneous epidemic of Gaertner infection among rats was found associated with methaemoglobinaemia and, in some cases, anaemia.
2. Strains of Gaertner's bacillus isolated from these rats reproduced methaemoglobinaemia in fresh rats but not in rabbits, guinea-pigs or mice.
3. Other strains of Gaertner's bacillus from rats, guinea-pigs and human sources also caused methaemoglobinaemia either before or after passage through rats.
4. Other organisms pathogenic for rats did not produce methaemoglobinaemia.

<sup>1</sup> Cf. W. J. Logie, *Journal of Pathology*, Vol. xv. (1910) p. 146; *Journal of Hygiene*, Vol. x. (1910) p. 143; Vol. xi. (1911) p. 361; E. Pelz, *Cent. f. Bakt. Orig.* Vol. LVII. (1910) p. 1; P. Mazé, *Compt. Rend.* Vol. clii. (1911) p. 1624.

THE INFLUENCE OF THE CULTURE MEDIUM ON  
THE GERMINATION OF ANTHRAX SPORES,  
WITH SPECIAL REFERENCE TO DISINFECTION EXPERIMENTS.

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IN the determination of the germicidal action of various disinfectants, it is customary, after treating emulsions of the organisms or of their spores, or silk threads which have been soaked in such emulsions, with the disinfectant agent for the required time, to test the vitality of the organisms or of the spores by inoculating traces of the treated emulsion or of the treated thread (with or without preliminary washing or other treatment) into broth culture media. If a growth ensues, presumably the organisms or the spores have not been killed; while if there be no growth, presumably the organisms or the spores have been killed.

One of us (R.T.H.), in testing the germicidal action of certain emulsified disinfectants of the higher coal-tar series, noticed that if similar sub-cultures of the treated material were made both in broth and on the surface of sloped agar, it was only with the weaker disinfectant solutions that a growth was obtained in broth; with stronger concentrations, no growth was obtained in broth sub-cultures, even after incubation for so long as nine days or more, while with the same material and concentrations growths were frequently obtained on surface agar. That is to say, while the broth cultures indicated that the anthrax spores had been killed by the particular solutions, the agar

cultures proved that this was not the case and that many of the spores still retained vitality. The broth used in the experiments was ordinary nutrient peptone broth<sup>1</sup> and was a perfectly satisfactory culture medium when inoculated directly with anthrax spores, always giving good growths in the controls, which were made for every experiment. That is to say, if broth is inoculated with a considerable number of untreated anthrax spores, growth ensues; when, however, the spores are either few in number, or are partially de-vitalised by a disinfectant, they do not as a rule develop in broth. These observations have induced us to make further experiments on this point.

#### PART I.

First of all the effect of five well-known potent emulsified disinfectants was tested on a suspension of anthrax spores. A virulent anthrax bacillus was grown on the surface of agar for (1)  $3\frac{1}{2}$  days and (2) three weeks, the growths were emulsified in sterile water so as to form a distinctly opalescent suspension when viewed in an ordinary test-tube (15 mm. diameter). The emulsions were filtered through sterile filter paper to remove masses, and one drop of the emulsion was added to each cubic centimeter of the various disinfectant solutions, of which 5 c.c. were employed for each test. Spores of two different ages were used because it is well known that old spores are more resistant than young spores.

The five disinfectants employed all have a Ridead-Walker carbolic coefficient of from 12 to 20 for the typhoid bacillus. Each disinfectant was employed in three different strengths, namely 2 per cent., 5 per cent. and 10 per cent. The time of exposure of the anthrax spores to the disinfectant was in some cases one hour, in others three days, and the temperature was maintained at about 20° C. After the period of exposure, broth, and surface agar, tubes were inoculated [each tube with one standard (3 mm.) platinum loopful], and were then incubated at 37° C. up to 72 hours if necessary. The results obtained are given in the Tables (I, II, and III) of the experiments at the end of this section. It will be seen from these that with the young anthrax spores ( $3\frac{1}{2}$  days) with an exposure of one hour to the action of the disinfectant solutions, the 2 per cent. solutions of all the disinfectants gave no growth in broth, but were all positive (*i.e.* gave a good growth) in the agar; with the 5 per cent. and 10 per cent.

<sup>1</sup> Whether made with "Lemco" or with meat seems to make no difference.

solutions no growth was obtained in either the broth or agar subcultures. (See Table I.) From this experiment it is obvious that had the broth cultures been taken as the index of the destruction of the anthrax spores, the 2 per cent. solutions would have been regarded as sufficient, whereas the agar cultures demonstrated that this was not the case. Still more striking were the results obtained with the older spores (*i.e.* three weeks old). Table II shows the results with an exposure of one hour to the 2 per cent. disinfectant solutions. It will be seen that *not one* broth culture gave a growth, while *all* the agar cultures were positive. Table III shows the results obtained with the old spores (three weeks old) with an exposure of no less than three days

TABLE I.  
Disinfectant allowed to act for one hour.  
Young anthrax culture used (3½ days old).

Disinfectant	Strength	Broth	Agar	Strength	Broth	Agar	Strength	Broth	Agar
A	2 0/0	-	+	5 0/0	-	-	10 0/0	-	-
B	2 0/0	-	+	5 0/0	-	-	10 0/0	-	-
C	2 0/0	-	+	5 0/0	-	-	10 0/0	-	-
D	2 0/0	-	+	5 0/0	-	-	10 0/0	-	-
E	2 0/0	-	+	5 0/0	-	-	10 0/0	-	-

- = no growth; + = growth of anthrax.

TABLE II.  
Disinfectant allowed to act for one hour.  
Old anthrax culture used (three weeks old).

Disinfectant	Strength	Broth	Agar	Strength	Broth	Agar	Strength	Broth	Agar
A	2 0/0	-	+	5 0/0	-	+	10 0/0	-	+
B	2 0/0	-	+	5 0/0	-	+	10 0/0	-	+
C	2 0/0	-	+	5 0/0	-	+	10 0/0	-	+
D	2 0/0	-	+	5 0/0	-	+	10 0/0	-	+
E	2 0/0	-	+	5 0/0	-	+	10 0/0	-	+

TABLE III.  
Disinfectant allowed to act for three days.  
Old anthrax culture used (three weeks old).

Disinfectant	Strength	Broth	Agar	Strength	Broth	Agar	Strength	Broth	Agar
A	2 0/0	-	+	5 0/0	-	+	10 0/0	-	-
B	2 0/0	-	+	5 0/0	-	+	10 0/0	-	-
C	2 0/0	-	+	5 0/0	-	+	10 0/0	-	-
D	2 0/0	-	+	5 0/0	-	-	10 0/0	-	-
E	2 0/0	-	+	5 0/0	-	+	10 0/0	-	+

at room temperature ( $17^{\circ}\text{ C.}$  to  $20^{\circ}\text{ C.}$ ). Again, not a single broth culture gave a growth, whereas all the agar sub-cultures with the 2 per cent. solutions were positive; with the 5 per cent. solutions, only Disinfectant D was negative, but with the 10 per cent. solutions all were negative except Disinfectant E.

## PART II.

Under regulation of the British Home Office, horse hair from certain countries, *e.g.* China, Siberia, Russia, may only be manipulated after being submitted to some process of disinfection, either to steam disinfection, or to some disinfecting agent "under conditions of concentration and temperature of the disinfectant, and duration and manner of exposure of the material to it, and otherwise, as are certified to secure the destruction of anthrax spores in all parts of all horse hair subjected to the process" (Factory and Workshop Orders 1901. For the use of Horse hair, 1907, No. 984).

"Certified" in the above means certified by the head of a Bacteriological Laboratory, and the Home Office accepts no responsibility for such certification.

We have tested two of the processes certified as sufficient under the above regulations. The first one tested specifies immersion of the material in a solution consisting of one part of Disinfectant A in 100 parts of water at a minimum temperature of  $15.5^{\circ}\text{ C.}$  and a minimum duration of exposure of one hour; large bundles of material to be opened out. The second one tested specifies immersion in a solution consisting of 0.5 per cent. of soft soap, 0.5 per cent. sodic carbonate and Disinfectant B, I in 120: minimum temperature  $20.2^{\circ}\text{ C.}$ , minimum duration of exposure one hour; large bundles of material to be opened out and soaked in water previous to treatment. We have submitted these processes to experimental investigation. Silk threads and horse hair, in the form of single hairs, and small pledges of unwashed sheep's wool, after preliminary sterilisation by heat, were soaked in aqueous emulsions of anthrax spores of varying age, and were then dried in dishes at  $37^{\circ}\text{ C.}$  The materials thus prepared were employed in the experiments detailed below.

*Process No. 1.**Disinfectant A, 1 % solution, acting for one hour.*

(a) HORSE HAIR. Soaked in a suspension of 12-days-old anthrax spores, dried for ten minutes, and placed in the disinfectant solution for one hour at 20° C. The hairs were then placed in broth and agar tubes and allowed to remain in the 37° C. incubator for 18 hours.

Result { Agar +  
          \ Broth -

Result after 72 hours' incubation same as above.

(b) WOOL. Same procedure as in (a) but wool not allowed to remain on agar, only passed over the surface. Wool allowed to remain in broth.

Result { Agar -  
          \ Broth -

(c) SILK. Soaked in a suspension of four-days-old anthrax spores. Procedure as in (a).

Result { Agar +  
          \ Broth -

(d) SILK. Soaked in a suspension of 12-days-old culture spores. Procedure as in (a).

Result { Agar +  
          \ Broth -

(e) HORSE HAIR. Soaked in a suspension of three-weeks-old anthrax spores. Procedure as in (a) except allowed to dry for 36 hours at 37° C.

Result { Agar +  
          \ Broth -

(f) WOOL. Soaked in a suspension of 12-days-old anthrax spores, then dried. Procedure as in (a).

Result { Agar -  
          \ Broth -

(g) WOOL. Soaked in a suspension of three-weeks-old anthrax spores. Procedure as in (a).

Result { Agar + (slight)  
          \ Broth -

(h) WOOL. Soaked in a suspension of three-weeks-old anthrax spores. Disinfectant was then allowed to act for one hour, after which the wool was thoroughly dried, and finally placed in agar and broth tubes.

Result { Agar +  
          \ Broth -

*Disinfectant A, 1 % solution, acting for two hours.*

(i) SILK. Soaked in a suspension of 12-days-old anthrax spores; disinfectant then allowed to act for two hours at 20° C.

Result { Agar +  
          \ Broth -

(j) HORSE HAIR. Soaked in a suspension of 12-days-old anthrax spores. Procedure as in (i).

Result { Agar +  
          \ Broth -

Disinfectant A, 2 % solution, acting for one hour.

(k) HORSE HAIR. Soaked in a suspension of three-weeks-old anthrax spores; disinfectant then allowed to act for one hour at 20° C. Treated hair then inoculated on to slope agar and allowed to incubate for 18 hours at 37° C.

Result. Agar +

(l) SILK. Soaked in a suspension of three-weeks-old anthrax spores. Procedure as in (k).

Result. Agar +

Disinfectant A, 2 % solution, acting for two hours.

SILK. Soaked in a suspension of 12-days-old anthrax spores; disinfectant then allowed to act for two hours at 20° C. Treated silk finally inoculated on to slope agar tubes and allowed to incubate for 18 hours at 37° C.

Result. Agar +

#### *Process No. 2.*

Disinfectant B solution.

(a) SILK. Soaked in a suspension of 12-days-old anthrax spores; disinfectant then allowed to act for one hour at 20° C. Agar and broth tubes then inoculated and incubated at 37° C. for 18 hours.

Result { Agar +  
          | Broth -

After 48 hours' incubation,

Result { Agar +  
          | Broth +

(b) WOOL. Soaked in a suspension of 12-days-old anthrax spores, then allowed to dry in 37° C. incubator overnight; disinfectant then allowed to act for one hour at 20° C., wool dried again (as before) and finally inoculated into broth and agar tubes.

Result { Agar +  
          | Broth -

(c) HORSE HAIR. Soaked in a suspension of three-weeks-old anthrax spores. Procedure as in (a).

Result { Agar +  
          | Broth -

After 48 hours' incubation,

Result { Agar +  
          | Broth +

(d) SILK. Soaked in a suspension of four-days-old anthrax spores. Procedure as in (a).

Result { Agar +  
          | Broth +

(e) WOOL. Soaked in a suspension of three-weeks-old anthrax spores. Procedure as in (a).

Result { Agar +  
          | Broth -

From the foregoing experiments it will be seen that neither 1% solution of Disinfectant A nor the Disinfectant B solution is effective in destroying anthrax spores. The difference is also well brought out between agar and broth media as agents for testing the vitality of the spores, the agar cultures being almost always positive, while the broth cultures were generally (only three exceptions) negative. Even a 2 per cent. solution of Disinfectant A acting for two hours (double the concentration and time recommended) was inefficient to destroy the anthrax spores.

### PART III.

Recently a method has been devised by Mr Seymour-Jones for the disinfection of anthrax-infected skins and hides. It consists in soaking the skins for 24 hours in a solution containing 1% formic acid (90 per cent. strength) to which is added mercuric chloride sufficient to make a 1-5000 solution. After this treatment the skins are transferred for an hour or so to a saturated solution of common salt in water. This process has been the subject of an exhaustive investigation by Constant Ponder<sup>1</sup>, who reports favourably upon it. We have tested the process on artificially infected material, soaking the material after treatment with the disinfecting solution in ammonium sulphide so as to render inert any adherent mercuric chloride. The result has been that silk threads and horse hair give no growth on agar or in broth, but sheep's wool has invariably given growth on agar (but not in broth), showing that the anthrax spores had not been killed.

It is difficult to explain why the wool is not disinfected, while the silk threads and hair are disinfected, by this process. It may be that the disinfecting solution is unable to penetrate into the interior of the wool pledges on account of adherent grease. We thought that entangled air might play a part, but the results were the same even if the vessels are partially exhausted of air, so that the entangled air-bubbles are removed.

### SUMMARY.

The foregoing experiments conclusively show that a broth medium is quite unsuitable as a test culture medium to determine the vitality of anthrax spores in disinfection experiments, whereas agar is a suitable

<sup>1</sup> A report to the Worshipful Company of Leather-sellers on the incidence of anthrax amongst those engaged in the hide, skins and leather industries, with an inquiry into certain measures aiming at its prevention. Published by the Worshipful Company of Leather-sellers, London, 1911.

and delicate medium for the purpose, even when considerable traces of the disinfectant are carried over with the inoculation.

The reason for this inefficiency of broth is not obvious. We thought that it might be due to the absence of bacillary forms in the sporing material, but the emulsion of spores heated to 80° C. for 15 minutes and then inoculated directly into broth gave good growths.

Absence of oxygen might be another factor, but the results were the same when splinters of sterilised wood infected with anthrax spores were treated. The wood floated on the surface of the broth and so was subjected to a free supply of oxygen, yet no growths were obtained in broth when the splinters were soaked in the disinfectants, while good growths were obtained on agar. The control splinters gave good growths in broth. It may be that the anthrax spores are partially de-vitalised by the action of the disinfectant and that in this condition broth is a comparatively unsuitable culture medium for them. Prolonging the time of incubation of the broth cultures up to 10 or 14 days makes no difference. If a culture in broth shows no growth in 48 hours, a growth hardly ever appears with more prolonged incubation. Nor is this superiority of agar over broth as a culture medium confined to the emulsified disinfectants employed in these experiments, for similar results have been obtained with phenol, and with formaldehyde, the latter both in the fluid (formalin), and in the gaseous, conditions.

## PURIFICATION OF WATER BY INFUSORIA.

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### *Introduction.*

ACCORDING to Hüntemüller (1905), Razetto (1907), Stokvis (1909) etc. flagellates contribute actively to the extermination of bacteria in water. River water, naturally polluted by bacteria or artificial bacterial emulsions (of *Bacillus typhosus*), could be cleared in four days if flagellates (*Bodo saltans* or *B. ovatus*) were added to it.

The object of our present research was: (1) to see whether other Protozoa especially Infusoria have the same bactericidal power, (2) to study some of the conditions favouring or inhibiting this power.

We made our experiments by adding Infusoria (*Colpoda cucullus*) to emulsions of bacteria (*B. typhosus* and *Vibrio cholerae*). The Infusoria were obtained from cultures in hay-infusion, where they were living together with *B. subtilis*, *B. megatherium* and *Spirilla*. The emulsions were obtained from pure cultures aged 24 hours and cultivated at a temperature of 37° C.

*Demonstration of the bactericidal power of Colpoda cucullus.*

Our first experiment served to ascertain whether *C. cucullus* shows the same bactericidal power as the flagellates studied by our predecessors. We prepared four bottles, two of them containing an emulsion of *B. typhosus*, the two others an emulsion of *V. cholerae*. To each of the bottles containing the bacteria was added a loopful of a culture of *Colpoda*, then the bottles were placed at a temperature of 22° C. Table I shows the results of this experiment.

TABLE I.

Date	Emulsions of <i>B. typhosus</i> with <i>Colpoda</i>	Emulsions of <i>V. cholerae</i> with <i>Colpoda</i>	Emulsions of <i>B. typhosus</i>	Emulsions of <i>V. cholerae</i>
June 14	Fluid clouded ...	Fluid clouded ...	Fluid clouded	Fluid clouded
.. 15	Fluid clouded; few <i>Colpoda</i> present	Fluid clouded; few <i>Colpoda</i> present	"	"
.. 16	Fluid begins to clear; many <i>Colpoda</i> present	Fluid clouded; many <i>Colpoda</i> present	"	"
.. 17	Fluid almost cleared; many <i>Colpoda</i> present	Fluid begins to clear; many <i>Colpoda</i> present	"	"
.. 18	Fluid completely cleared; no <i>Colpoda</i> present	Fluid almost cleared; many <i>Colpoda</i> present	"	"
.. 19	Fluid completely cleared; no <i>Colpoda</i> present	Fluid completely cleared; no <i>Colpoda</i> present	"	"

This table shows, that the *Colpoda* are able to clear away emulsions containing *B. typhosus* and *V. cholerae* and that this clearance is preceded by an increase in the number of Infusoria. After the disappearance of the bacteria the number of *Colpoda* rapidly diminishes.

On repeating this experiment with *Vibrio Dunbar*, *V. El Tor*, a water-vibrio resembling *V. El Tor*, *B. megatherium* and *Spirillum volutans*, the emulsions containing these microbes were all cleared after 4–5 days when *Colpoda* were added to them.

The Infusoria used in these experiments were always mixed with bacteria, especially with *B. subtilis*. It might be asked whether the clearing of the emulsions is not due to some noxious influence which *B. subtilis* may exert on the bacteria present in the emulsions. This supposition is however untenable since we were unable to clear the emulsions with the aid of cultures of bacteria found together with the *Colpoda*.

Hüntemüller proved that the flagellates kill the bacteria by phagocytosis. Some authors think however that they do so by the production of toxic excretions. It is impossible to demonstrate bacilli within the Infusoria, because they are rapidly destroyed within the nutrient vacuoles. Consequently we do not know whether phagocytosis actually takes place. To ascertain whether the bacteria are killed by poisonous excretions of the Infusoria, a culture of *Colpodae* was filtered through a Chamberland-filter; 5 c.c. of the filtrate, which was completely clear and did not contain any traces of Infusoria, was added to an emulsion of *V. cholerae*. . . .

After two weeks no sign of clearing was to be seen. It may be added that the same negative result was obtained when using the filtrate of a flagellate culture (*Monas* sp.), whereas this flagellate itself showed a marked bactericidal power. Consequently we may conclude that the bactericidal power of *C. cucullus* does not depend upon its excreting toxic substances.

Since the bactericidal action of Infusoria and Flagellates may be very effective in clearing river water it is of importance to determine the conditions influencing their power of destroying bacterial life. With this object we studied the influence of temperature, sunlight, oxygen and of some impurities found in river water upon the organisms in question.

*Influence of temperature.* Bottles containing emulsions of *B. typhosus* and *V. cholerae*, to which *Colpodae* were added, were kept at temperatures of 20° C. and 6° C. Table II shows the results of this experiment.

TABLE II.

Date	Emulsions of <i>B. typhosus</i> at 20° C.	Emulsions of <i>V. cholerae</i> at 20° C.	Emulsions of <i>B. typhosus</i> at 6° C.	Emulsions of <i>V. cholerae</i> at 6° C.
June 10	Fluid clouded . . .	Fluid clouded . . .	Clouded	Clouded
" 12	Fluid begins to clear . . .	" " . . .	" "	" "
" 13	Fluid almost cleared . . .	Fluid begins to clear . . .	" "	" "
" 17	Fluid completely cleared	Fluid almost cleared . . .	" "	" "
" 19	" " "	Fluid completely cleared	" "	" "
" 20	" " "	" " "	" "	" "

Consequently no bactericidal influence was to be observed at a temperature of 6° C. Similar negative results were obtained at a temperature of 37° C.

*Influence of oxygen.* Emulsions of *B. typhosus* and *V. cholerae* + *Colpodae* were kept under aerobic and anaerobic conditions.

The following results were obtained (Table III):

TABLE III.

Date	Emulsions of <i>B. typhosus</i> , aerobic cultures	Emulsions of <i>V. cholerae</i> , aerobic cultures	Emulsions of <i>B. typhosus</i> , anaerobic cultures	Emulsions of <i>V. cholerae</i> , anaerobic cultures
June 16	Fluid clouded ...	Fluid clouded ...	Clouded	Clouded
" 19	Fluid almost cleared ...	Fluid begins to clear ...	"	"
" 20	Fluid completely cleared	Fluid almost cleared ...	"	"
" 27	" " "	Fluid completely cleared	"	"

Consequently no bactericidal effect was observable under anaerobic conditions.

*Influence of sunlight.* Emulsions of *B. typhosus* and *V. cholerae* + *Colpodae* were exposed to the influence of direct sunlight for  $1\frac{1}{2}$  hours.

The following results were obtained (Table IV):

TABLE IV.

Date	Emulsions of <i>B. typhosus</i> in the dark	Emulsions of <i>V. cholerae</i> in the dark	Emulsions of <i>B. typhosus</i> in sunlight	Emulsions of <i>V. cholerae</i> in sunlight
June 14	Fluid clouded ...	Fluid clouded ...	Fluid clouded ...	Fluid clouded
" 16	Fluid begins to clear	Fluid almost cleared	Fluid almost cleared	Fluid begins to clear
" 17	Fluid almost cleared	Fluid completely cleared	Fluid completely cleared	Fluid almost cleared
" 19	Fluid completely cleared	Fluid completely cleared	Fluid completely cleared	Fluid completely cleared

Consequently direct sunlight has no influence on the bactericidal power of the Infusoria. It is interesting, that in this experiment the emulsion of *V. cholerae* was cleared sooner than that of *B. typhosus*. Generally the latter emulsion is the first to be cleared.

*Influence of impurities in water.* Emulsions of *B. typhosus* and *V. cholerae* were made with water from the canals running through Amsterdam. This water is very impure as it receives the contents of the sewers, moreover it is brackish. Before using it to make the emulsions it was filtered through a Chamberland-filter. Table V shows the results of this experiment.

TABLE V.

Date	Emulsions of <i>B. typhosus</i> in tapwater	Emulsions of <i>V. cholerae</i> in tapwater	Emulsions of <i>B. typhosus</i> in canal-water	Emulsions of <i>V. cholerae</i> in canal-water
June 14	Fluid clouded ...	Fluid clouded ...	Fluid clouded ...	Fluid clouded
" 16	Fluid begins to clear	" " "	" " "	" "
" 17	Fluid almost cleared	Fluid begins to clear	Fluid begins to clear	" "
" 19	Fluid completely cleared	Fluid completely cleared	Fluid completely cleared	Fluid completely cleared

Consequently we note a slight delay (of about 24 hours) in the clearance of the emulsions made with canal-water.

The sewage of starch manufactories, containing a considerable quantity of amyllum, has a markedly unfavourable influence on the bactericidal power of Infusoria. We imitated this sewage by using an infusion of potatoes. With this infusion, filtered before use, we prepared emulsions of *B. typhosus* and *V. cholerae*. The emulsion to which *Colpodae* were added was not cleared after two weeks.

A similar unfavourable influence was observed by preparing the emulsions with water containing ammonium chloride and other ammonium salts such as occur in sewage derived from gas factories.

#### SUMMARY.

Infusoria have the same bactericidal power as flagellates. Emulsions containing *Bacillus typhosus*, *Vibrio cholerae*, *V. Dunbar*, *V. El Tor*, *B. megatherium* and *Spirillum volutans*, to which *Colpoda cucullus* is added, are soon cleared. Before the clearance the *Colpodae* multiply actively.

This bactericidal effect does not depend upon the production of toxic substances by the Infusoria. The fluid obtained from filtered cultures of the Infusoria exerts no bactericidal effect. Only living *Colpodae* are able to clear the emulsions.

Direct sunlight does not prevent the clearing of the emulsions by the Infusoria, but temperatures below 10° C. and above 30° C. and absence of oxygen (anaerobic culture) are unfavourable to their exerting a bactericidal effect.

The polluted water of the canals of Amsterdam slightly delayed the clearance of the emulsions; the sewage of starch factories and gas factories completely prevented this clearance.

Consequently under natural conditions Infusoria will only play a part in purifying river water (1) if the temperature is above 10° and under 30° C., (2) if the aquatic vegetation is rich enough to supply the necessary quantity of oxygen, (3) if the water is not highly polluted by adjoining factories.

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## FURTHER EXPERIMENTS ON VARIABILITY IN THE GAS-FORMING POWER OF INTESTINAL BACTERIA.

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(With 5 Diagrams and 2 Charts.)

IN a recent communication (Penfold 1911) I described experiments with three organisms, *B. coli* Escherich, *B. enteritidis* Gaertner and *B. Grünthal*, showing that limitation or complete abolition of certain gas-forming powers normally possessed by these organisms, could be brought about by their growth on chloracetic acid-agar, and further, that in the case of the latter organism, resistance to this medium was associated with such limitation or abolition.

In this paper I desire to give an account of further experiments dealing with the same subject. The same special medium and technique were employed as detailed in the paper cited above. Further experiment had shown that *B. lactis aerogenes* when grown for seven successive generations on chloracetic acid-agar, still gave at the end of the period a good yield of gas on glucose, although it was highly resistant to the chloracetic acid medium; similarly a strain of *B. acidi lactici* gave negative results in spite of long training.

In view of the anomalous behaviour of these organisms it appeared desirable to examine more fully the nature of the selective process in those cases in which non-gas-producing variants had been so easily obtained.

### *Further experiments with B. coli Escherich.*

Chloracetic acid-agar plates of this organism show, at suitable concentrations of the salt, mixtures of big and little colonies, that is, the growth of all the organisms is not inhibited equally; at higher concentrations only the big colonies grow. In the successful experiments described in my first paper, the non-gas-producing strains were

obtained by selecting big colonies in each case. Further work has shown me that the association of non-gas-production and resistance to chloracetic acid is in the case of *B. coli* Esch. a very variable one. Some essential connection exists between the two properties, but this connection could be disclosed only by a prolonged statistical enquiry. The following experiment illustrates this point:

A pure culture of normal *B. coli* Esch. was plated out on 12 plates of chloracetic acid-agar. These plates were divided into four groups containing different concentrations of sodium chloracetate, each member of a group of three containing the same concentration of this salt.

The respective concentrations were 0·01 %, 0·05 %, 0·1 % and 0·2 % expressed in terms of the amount of acid added. The three plates of the latter concentration showed only large colonies. Five large colonies from each of the 0·2 % plates were inoculated into glucose-peptone-water in Durham tubes; these tubes were observed daily for five days and the maximum amounts of gas found were as indicated in the following table:

TABLE I.

Colonies from plate D	1/3	1/3	1/3	1/2	5/12
" " H	1/4	nil	1/2	1/2	1/2
" " L	7/12	7/12	5/12	1/2	1/2

These fractions indicate the length of gas tube displaced by gas.

Control tubes of glucose-peptone-water, inoculated from the same original strain of *B. coli* grown on MacConkey plates, gave in the case of twenty different colonies the following volumes of gas:

4 tubes yielded	gas equal to	1/2	of the gas tube.
12 "	"	7/12	"
4 "	"	2/3	"

The colonies from the chloracetic agar plates, therefore, show one giving no gas, six others below the lowest of the controls, and eight similar to the controls.

The strain from plate H, which gave no gas on glucose-peptone-water, yielded, when tested on lactose-peptone-water, 1/12th of a tubeful of gas. It was therefore plated out on a lactose plate and eight of its colonies were tested again on lactose-peptone-water, when seven of these were found to produce no gas while the remaining one showed half-a-tubeful. The large colonies, apparently, do not appear to be homogeneous in their composition.

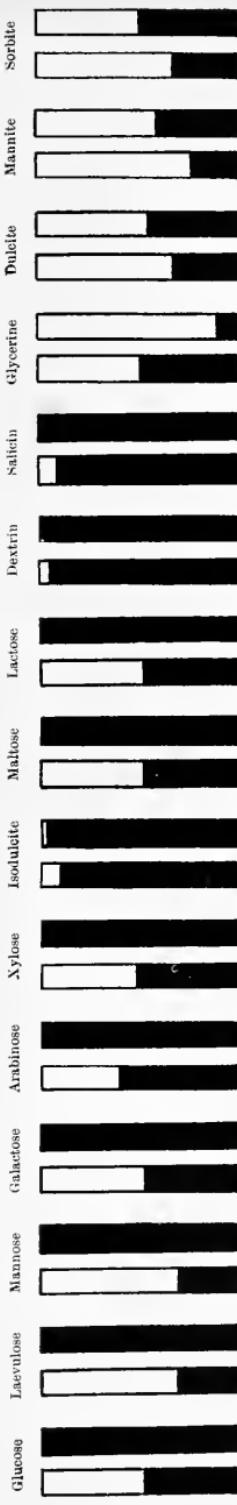


Diagram I. *Bacillus coli communs* Escherich, strain *II*. Left-hand member of couple = normal strain; right-hand member of couple = variant strain. In this diagram the black columns represent gas tubes containing no gas, while in the other columns the white spaces represent the quantities of gas formed. All the tubes were incubated for five days at 37° C., and the amount of gas indicated in the diagram is the greatest amount found in the individual tubes during the whole period. The tubes are arranged in couples, the left-hand member representing the normal *Bacillus coli*.

One of the seven lactose broths which gave no gas (denoted by *H*) was subcultured on agar and tested on the usual peptone-waters containing different carbohydrates. Diagram I represents its behaviour in respect of gas-formation on the carbohydrates which it fermented.

This strain "*H*" is a more striking example of the loss of the power to form gas from sugars, than the strain which the writer described in the formerly published paper dealing with gas variants. In the former case the organism still produced traces of gas from galactose, xylose, arabinose, isodulcite, maltose and salicin, whereas this new strain produced only a slight trace of gas in isodulcite, out of nine sugars tested. The essential difference between the selective processes in the two cases was, that in the former strain a large colony was taken off the first chloracetic agar plate haphazard, and replated on the same medium, from which again a single colony was picked off and replated, the process being repeated four times, while in the case of the latter strain, a large number of colonies were tested from the first chloracetic agar plates, with the result that one was found which from the start failed to give any gas on

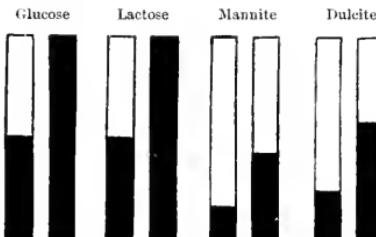


Diagram II. *B. coli* Escherich. Left-hand member of couple = normal strain; right-hand member of couple = variant strain.

glucose. In order to make certain that this was not a matter of chance, the same method was repeated again with the same organism. In this case 17 colonies were inoculated into glucose broth, from chloracetic agar plates which had been incubated five days at 37° C. All the broths showed a full acid reaction after 24 hours, but after 48 hours two of the tubes showed no gas. On this date one of the two tubes got broken but the other showed no gas throughout an observation period of six days. This new strain gave the reactions shown on Diagram II. It would appear, therefore, that the easiest and best way to get these variants is by testing a large number of colonies from the first chloracetic agar plates.

*The examination of the new variety in Theobald Smith's tubes.*

The above-mentioned strain "H" was compared with the normal strain from which it was derived, in Smith's tubes, and the results obtained are recorded in Table II. The new strain, it will be seen, gave no gas in glucose but its yield in mannite broth was only 50% of the normal, a diminution distinctly greater than was found with the Durham tube (see Diagram I).

TABLE II.

*Theobald Smith's tubes.*Strain "H" versus Normal *B. coli* Esch.

	Normal <i>B. coli</i> .		Varied <i>B. coli</i> .	
	Glucose		Glucose	
	1st tube with 20 divisions	2nd tube with 10 divisions	Tube with 20 divisions	Tube with 10 divisions
Vols. of gas 1st day 10 div.	4·8 div.	nil	nil	nil
" 2nd , 11 "	4·8 ..	nil	nil	nil
" 3rd , 11 "	4·8 ..	nil	nil	nil
" 5th , 10 "	4·2 ..	nil	nil	nil
Mannite				
	Tube with 20 divisions	Tube with 5 divisions	Tube with 20 divisions	Tube with 5 divisions
Vols. of gas 1st day 8·5 div.	2·2 div.	4·5 div.	1·4 div.	
" 2nd , 13·5 "	3·4 ..	7·0 ..	2·1 ..	
" 3rd , 14·5 "	4·0 ..	7·2 ..	2·1 ..	

*Further experiments with *B. enteritidis* Gaertner.*

The variant strain of *B. enteritidis* Gaertner described by the author in a former paper, while unable to produce gas from certain pentoses, was still able to produce a little gas from the hexoses. In order, therefore, to obtain strains unable to produce gas from glucose, it was deemed advisable to test a large number of colonies from the first chloracetic agar plates in respect of their action on glucose broth.

A typical strain of *B. enteritidis* was plated on the special medium on a series of plates: the strength of chloracetic acid in these plates varied from 0·1% to 0·9%. These plates were grown for three days at 37° C. when colonies of large size were picked off and inoculated into glucose broth in order to test their gas-forming power. The results are given in Table III.

TABLE III.

Strength of chloracetic acid in the plates	Number of large colonies tested	Extreme deviation of gas yield, in fractions of gas tube
0·1 %	4	1/3 to 5/12
0·3 %	4	1/4 to 5/12
0·4 %	4	1/3 to 5/12
0·5 %	4	1/12 to 1/3
0·7 %	4	1/6 to 5/12
0·9 %	4	1/4 to 5/12

Here we have 24 colonies none of which give less than one-twelfth of a tubeful of gas. The same plates were, therefore, further incubated until the tenth day, when the large colonies were again inoculated into glucose-peptone-water and gave the gas yields seen in Table IV.

TABLE IV.

Strength of chloracetic acid in the plates	Number of large colonies tested	Extreme deviation of gas yield, in fractions of gas tube
0·1 %	8	1/12 to 5/12
0·3 %	8	Pin point bubble to 1/12
0·5 %	8	" " " 5/12
0·7 %	8	Nil to 5/12
0·9 %	8	Nil to 1/12

It appeared, therefore, that between the third and the tenth day the character of these colonies had altered, the organisms of which they were composed having become distinctly poorer gas producers. The gas-producing elements in the colonies had probably been killed off or outgrown by the non-gas-producing ones. The fact remains that no less than three of the plates showed big colonies able to produce amounts of gas nearly equal to those produced by the normal strains. Out of the total of 40 colonies tested, only three showed themselves unable to produce gas in glucose broth, while no less than six gave an amount of gas just equal to a pinhead bubble. Three tubes out of the 40 showed 5/12ths of a tubeful of gas, i.e., an amount but little different from the normal. It is important to notice, that in spite of these very different gas yields, all the colonies agreed in having grown to a large size on the special medium. Two of the three glucose tubes which gave no gas had subcultures taken from them on to agar slopes. From the latter they were put through the different carbohydrate broths with results shown in Diagram III.

Both these new strains produced no gas on either glucose or maltose but still gave very fair yields from the alcohols; the retention of the slight gas-producing power of xylose is rather curious, and in this respect they differ from the strain of which an account was published in my previous communication.

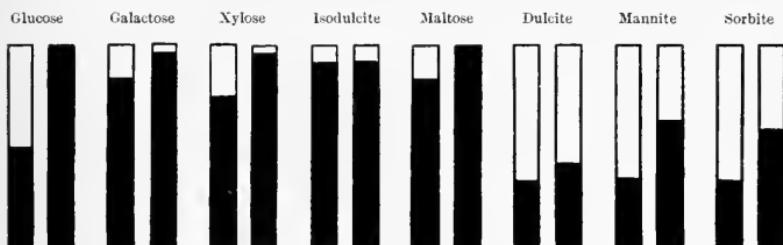


Diagram III. *Bacillus enteritidis* Gaertner. Left-hand member of couple = normal strain; right-hand member of couple = variant strain.

#### *Further experiments with B. paratyphosus B.*

A standard laboratory strain of this organism was plated on chloroacetic acid-agar plates, in which the strength of chloroacetic acid varied from 0·1% to 0·9%. Large and small colonies developed. After three days' growth 14 large colonies from the plates were tested on glucose-peptone-water. All showed some gas production. After seven days' further incubation 20 large colonies were tested on glucose-peptone-water, when no less than 14 of them were found to produce no gas. Five of these were put through various carbohydrates, but none of

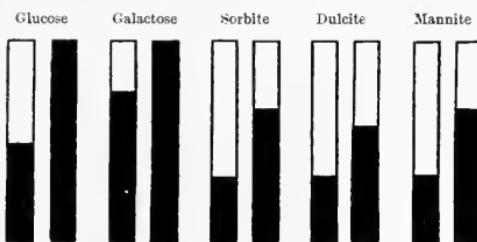


Diagram IV. *B. paratyphosus* B. Left-hand member of couple = normal strain; right-hand member of couple = variant strain.

them was able to produce gas from glucose or maltose. One of the strains tested on galactose produced no gas while they all produced gas from the alcohols they fermented. An idea of the amount of gas production on alcohols is given in Diagram IV in the case of one of the strains. The others were very similar.

*The occurrence of natural variants of the Gaertner group in respect of gas formation.*

I am indebted to Dr Bainbridge for numerous strains belonging to this group, two of which deserve mention in this paper.

1st. A non-gas-producing *B. Aertryck*. This gave all the cultural and serological reactions proper to this variety, except that it failed to produce gas from any sugar.

2nd. A strain of *B. paratyphosus* B. (Murray) which produced very little gas on any medium. This organism was irregular in its action on dulcite broth but apart from this, and its poor gas-producing power, it was a definite strain of *B. paratyphosus* B. Moreover it had been isolated from a definite case of paratyphoid fever. This strain was grown on chloracetic acid-agar with a view to removing its gas-forming power, and the normal and selected strains are compared in this respect in Diagram V.

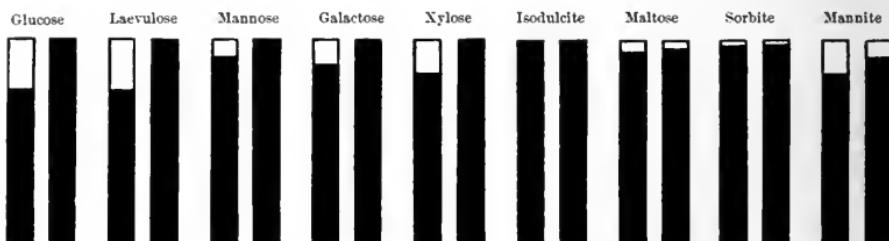


Diagram V. Strain "Murray," *B. paratyphosus* B. Left-hand member of couple = normal strain; right-hand member of couple = variant strain.

Here again the power to form gas from sugars disappeared except in the case of maltose; the power to produce gas from mannite and sorbite is retained to a slight extent, but for which fact it would have approached very closely to the naturally occurring non-gas-producing *B. Aertryck* above described. The agglutination characters of this organism were not altered in the process of selection.

*Morgan's bacillus No. 1.* This organism by growth on chloracetic acid-agar lost the power to produce gas from glucose and laevulose, though it produced a good acid reaction in the medium overnight. On galactose no acid reaction was produced by the new strain until the sixth day, and then, curiously enough, a tenth of a tubeful of gas appeared. This was repeated and both these effects were obtained.

It seems therefore highly probable that this selective process for obtaining gas variants will be fairly generally successful if thoroughly applied. To get successful results, it is advisable to test a large number of colonies in the first instance and to allow the plates to incubate for 7-10 days before testing the colonies.

*A comparison of the gas-forming power of the big and little colonies taken from the same chloracetic-agar plates.*

*B. enteritidis* Gaertner was plated out on a series of plates containing ascending concentrations of chloracetic acid. After three days' incubation four big and four little colonies from each plate were subcultured into glucose broth when the average yield of gas was found to be as follows. (The results are expressed as tube divisions filled with gas.)

	Big colonies	Little colonies	Ratio
0·1 % plate	4·8	6·5	0·73
0·3 "	4·25	5·6	0·75
0·4 "	4·75	5·5	0·86
0·5 "	2·4	4·1	0·58
0·7 "	3·8	6·0	0·63
0·9 "	3·0	7·5	0·4

*B. paratyphosus* B. treated similarly gave the following figures:

	Big colonies	Little colonies	Ratio
0·1 % plate	2·6	2·5	1·0
0·2 "	1·3	2·8	0·46
0·3 "	1·0	3·1	0·32
0·7 "	0·5	2·6	0·19
0·8 "	1·0	3·2	0·3
0·9 "	0·5	2·0	0·25

These ratios are plotted in the accompanying Charts I and II.

The figures show that even after three days' growth on the special medium the big colonies are poorer gas producers than the small ones, the latter being in point of fact normal. Twenty-four small colonies of *B. enteritidis* Gaertner gave an average of 5·8 divisions of gas against

five normal controls which average five divisions. The disparity between big and little colonies in respect of gas formation, holds much more strongly when the plates have been incubated for a longer period. The gas yield of the big colonies in the case of *B. enteritidis* Gaertner and *B. paratyphosus* B, after the plates have been incubated ten days, is roughly indicated in an earlier portion of this paper (see pp. 491-3).

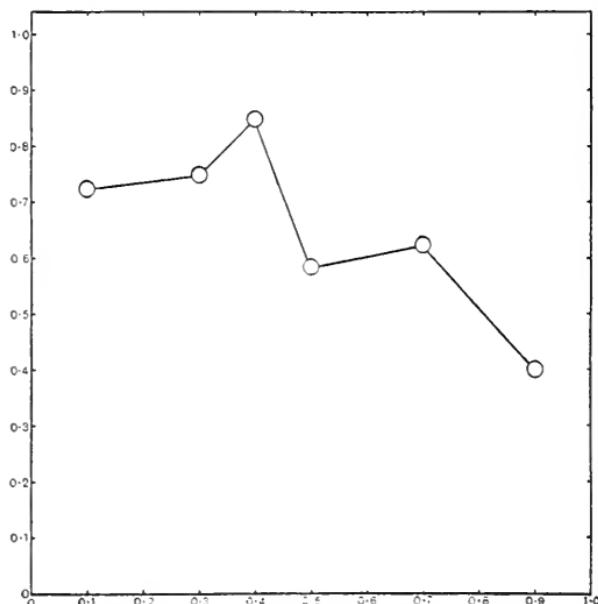


Chart I. *B. enteritidis* Gaertner. Ordinates represent ratios of average volumes of gas developed on glucose by big and little colonies respectively. Abscissae represent strengths of chloracetic acid in the plates.

Increased resistance to the chloracetic-acid medium is indicated by the increased size of some colonies, but in addition, these large colonies, in the later stages of their growth, show secondary colonies composed of organisms which appear to be more resistant to the medium than the average of the colony. In the case of *B. Grünthal*, an examination of some of these showed them to be poorer gas producers than the rest of the colony, and this agreed with the general principle that chloracetic acid resistance and poor gas-producing power are correlated. In the

case of *B. enteritidis* Gaertner, however, the papillae appear to be composed of organisms of similar gas-producing power to those found in the rest of the colony. This statement is based on the examination of about 40 tubes of glucose-peptone-water which had been inoculated, in equal proportions, from papillae and smooth interpapillary areas of colonies respectively. *B. paratyphosus* B. is similar to *B. enteritidis* Gaertner in this respect.

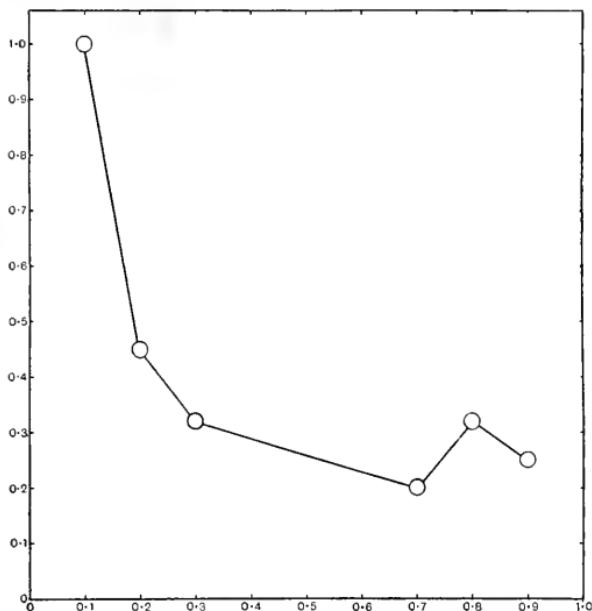


Chart II. *B. paratyphosus* B. Ordinates represent ratios of average volumes of gas developed on glucose by big and little colonies respectively. Abscissae represent strengths of chloracetic acid in the plates.

#### *Summary of foregoing experimental results.*

- (1) By the method of selection indicated in the paper it has been possible to obtain a variety of *B. coli* Escherich unable to produce gas from any sugar excepting isodulcite. The new strain so obtained was still able to produce gas from all the alcohols it fermented though in diminished amount.

(2) The power of *B. enteritidis* Gaertner to form gas from glucose and maltose has been removed in the same way. *B. paratyphosus* B. and *Morgan's bacillus* No. 1 have similarly given rise to variants not producing gas from the usual sugars.

(3) The easiest method to obtain non-gas-producing variants is by incubating plates of the respective organisms on the special medium for 7-10 days at 37° C. and then testing a fairly large number of large colonies on glucose-peptone-water.

(4) The relationship of chloracetic-acid resistance to non-gas-producing power is undoubtedly as between the big and little colonies of various strains, but it is of a complex character. The papillae in the case of *B. enteritidis* Gaertner and *B. paratyphosus* B. do not seem to differ materially from the rest of the colony bearing them, in gas-forming power.

#### *Durability of the new character.*

In my previous communication dealing with variation of the gas-producing power of these organisms, I showed that an artificially selected strain of *B. coli* Escherich, producing no gas from glucose, evinced no tendency to reversion though subcultured frequently on glucose-peptone-water.

The period was too short, however, to furnish much information in respect of permanency. I have now observed some of the new strains for a considerable period with the following results:

*1st Experiment.* *B. coli* Esch. In December 1910 a new strain of *B. coli* Esch. was selected, which produced no gas from glucose but 7/12ths of a tubeful when grown in mannite-peptone-water. From this period until May 1911 the strain was grown on ordinary agar, being subcultured on it about every fortnight. On May 19th, 1911, i.e., over five months after its selection, it was retested in respect of gas-producing power in glucose- and mannite-peptone-waters with the following result:

Date of observation	Glucose		Mannite	
	Reaction	Gas	Reaction	Gas
20th May	Acid	Nil	Acid	1/8
21st ,,	"	"	"	7/12
22nd ,,	"	"	"	7/12
24th ,,	"	"	"	7/12

*2nd Experiment.* Another strain of *B. coli* Esch. selected out shortly after the above, gave in May a bubble of gas the size of a pin's head when grown on glucose-peptone-water in a Durham tube.

*3rd Experiment.* An artificial strain of *B. coli* Esch. unable to produce gas from glucose in the Durham tube was grown anaerobically at 37° C. in glucose-peptone-water to which chalk had been added. Under anaerobic conditions it produced a small yield of gas and it was, therefore, thought to be reverting to its natural state. This anaerobic growth was continued for over one month. It was then plated out on lactose agar and three separate colonies were tested on glucose- and mannite-peptone-waters with the following result:

Glucose.									
Date of inoculation, March 28.			Colony...	1	2	3			
Observed	„	29	A.f.	G.n.	A.f.	G.n.	A.f.	G.	pinhead bubble
	„	31	„	„	„	„	„	„	„
April	1		„	„	„	„	„	„	„
	„	3	„	„	„	„	„	G.	bubble slightly larger

Mannite.									
Date of inoculation, March 28.			Colony...	4	5	6			
Observed	„	29	A.f.	G. 1/4	A.f.	G. 1/4	A.f.	G.	1/4
	„	31	„	G. 1/2	„	G. 1/2	„	G. 1/2	
April	1		„	G. 7/12	„	G. 7/12	„	G. 7/12	
	„	3	„	G. 1/2	„	G. 1/2	„	G. 1/2	

A.f. = Full acid reaction.

G.n. = Gas nil.

The gas appearing in the anaerobic culture was owing therefore only in the slightest degree to reversion; it was probably largely accounted for by the anaerobic growth and the presence of the chalk.

*4th Experiment.* *B. Grünthal*, by growth on the special medium and selection from it, had its gas-producing power in respect of lactose reduced from half a tubeful to a pinhead bubble. The strain was grown three months on agar and then plated. Twelve colonies were tested in lactose broth, when all were found to produce the same amount of gas as the strain did when first selected out.

*5th Experiment.* Strains of *B. paratyphosus* B. occasionally show a tendency to revert, in respect of gas-forming power on glucose, soon after being first selected out, but this tendency does not affect all the new strains. These new varieties have not been long under observation.

*Identity.*

The demonstration of the identity of the new strains has not been so strictly attended to in this series as in those described in my first communication on this subject where the evidence given was very complete. Since all the new varieties described in this paper show similar qualitative changes to those described in my previous communication it seemed unnecessary to devote so much effort in this direction.

*Testing of other chemical substances related to monochloracetic acid in respect of their action on the growth of intestinal bacteria.*

Marked variability in the size of colonies on plates, and the development of secondary colonies (*i.e.*, papillae formation), were especially looked for.

The organic acids used were all first neutralised in aqueous solution with saturated sodium carbonate solution, then filtered through a Berkefeld and added to agar plates in the usual way. Many of these bodies are unstable so that their temperature was kept as low as possible throughout the whole work. The chemicals used were all got from Kahlbaum except the potassium chlormalonate for which I was indebted to Miss Smedley of the Lister Institute.

*Monobromacetic acid.* This preparation inhibited the growth of *B. coli* Esch. and *B. enteritidis* Gaertner in weaker concentrations than monochloracetic acid. In the case of both organisms distinct variability in the size of the colonies was observed in suitable concentrations, but this was not so marked as with monochloracetic acid. Papillae appeared on the colonies, well developed in the case of *B. coli* Esch., but not so well developed in the case of *B. enteritidis* Gaertner. Fifteen large colonies from *B. enteritidis* and *B. coli* plates when tested on glucose showed no loss of gas-forming power. It was not found so easy to produce races highly resistant to this agent as had been the case with monochloracetic acid.

*α-Brompropionic acid.* In plates containing this agent *B. coli* Esch. produced small papillae on plates having a strength of 0·05% and 0·1%. Growth was not obtained on plates having a higher concentration than 0·3%. No marked variability in the size of the colonies was noted.

*B. enteritidis* Gaertner and *B. paratyphosus* A. showed, on agar containing this agent, no papillae formation nor marked variability in the size of the colonies.

Colonies from brompropionic acid plates of the above three organisms showed no variations from the normal strains in amount of gas produced from glucose.

Dibromsuccinic acid and the potassium salt of chlormalonic acid failed to select out variants of *B. coli* Esch. or *B. enteritidis* Gaertner. Sodium hippurate and benzoate were tried because of Herter's statement (1909) that they caused *B. coli* to produce less gas. Six concentrations of each salt, in agar plates, were used, from 0'01 % to 2 % in the case of the sodium benzoate, and from 0'02 % to 4 % in the case of the hippurate. In the case of *B. coli* Esch. and *B. Grünthal* no marked variability in the size of the colonies or naked-eye papillae formation was noted. Colonies from the plates of highest concentrations which showed growth were inoculated on the 11th day into glucose-peptone-water and produced normal amounts of gas.

Since the publication of my first communication on this subject Revis (1911) has described the production of an artificial variety from a typical *B. coli*. This variety differed from the normal by being unable to produce gas from any carbohydrate on which it was tested; it was, moreover, unable to clot milk. It was produced by the growth of the normal strain on malachite green media. Revis does not exclude the possibility of contamination arising during the course of the 15 subcultures necessary to produce the new variety. It is precisely because of this difficulty that all points of resemblance between the original and the new strains should be collected, to help us to determine whether the new strain was actually derived from the old strain or not. I made no proviso, as Revis alleges, that before and after variation the agglutination character should remain the same. I believe however that if it does, it helps to establish the origin of the new variety. Of course variations of the agglutination property of the various organisms have been proved to occur, and the proof in this case has been largely furnished by the cultural and other characters, which have meanwhile remained constant. Had Revis proved the identity of the agglutination characters of the old and new organisms, it would have made the case more convincing. When many characters of an organism vary at once it is difficult to convince oneself that a foreign organism did not drop into the test tube during some portion of a long subculturing process and outgrow the strain from which one started. Since there are not

many remaining characters left for identification purposes, one can only produce conviction by repeating the process a number of times with the same result. I feel very little doubt about the variation process described by Revis, as it bears a strong resemblance to the one described in the previous communication I made on this subject, and in that case the identity was determined by serum tests as well as by the standard cultural tests. Moreover other organisms varied similarly in the same environment.

#### GENERAL CONCLUSIONS.

1. The results of this research show that organisms of the coli-group can have various gas-forming powers removed by a process of artificial selection on a specially prepared medium. The biochemical properties of the variant strains approach closely those of the naturally occurring anaerogenes-coli class.

2. The power of gas-formation from sugars (always excepting isodulcite) may be lost when gas-formation from alcohols is retained. It is probable, therefore, that two different fermentations are engaged in the respective processes.

3. The research raises the question as to the weight to be attached to the power of fermenting glucose and lactose with gas-formation, in recognising *B. coli* in routine examinations of pathological material, water, foods, etc. Hitherto, in all authoritative catalogues of the necessary properties of this organism, this has been included but it probably ought to be regarded as not absolutely essential.

In conclusion I desire to express my indebtedness to Dr Ledingham for valuable help in the course of this research.

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## SOME NOTES ON INDOLE-REACTION AND ALLIED PHENOMENA.

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(With 6 Charts.)

### PART I.

By H. SEIDELIN.

A SHORT time ago I (1911) described a method for the quantitative estimation of indole. It may be of interest to reproduce the curves obtained by the use of this method, in the study of several strains of *Bacterium coli*, as curves representing indole-production have seldom been published.

The curves given by Marshall (1907) are based upon a small number of estimations, none of which was made later than the 23rd day, if we consider *B. coli* only; maxima were found, in the case of this bacterium, on the 5th and the 14th day, respectively. The estimations are much more quickly carried out according to my technique than by the method employed by Marshall; I was therefore able to make their number comparatively large.

For the purpose of studying the variations in the quantity of indole, a large number of peptone-water tubes were prepared at the same time, each tube containing 10 c.c. of 1% peptone (Witte) solution with  $\frac{1}{2}\%$  of NaCl. Some of the estimations have however been made in 2% peptone-water tubes, but the differences are of no importance, as the estimations in question were made after several weeks, whilst noticeable differences, depending on the strength of the peptone solution, were

in various tests found present during the first few days only. The tubes were inoculated with one loop each of a peptone-water culture of the corresponding strain and incubated at 37° C. Subsequently one tube was taken out every day and the quantity of indole determined. In this way the quantity of liquid in the tube diminishes, of course, by evaporation from day to day, and it is quite possible that this

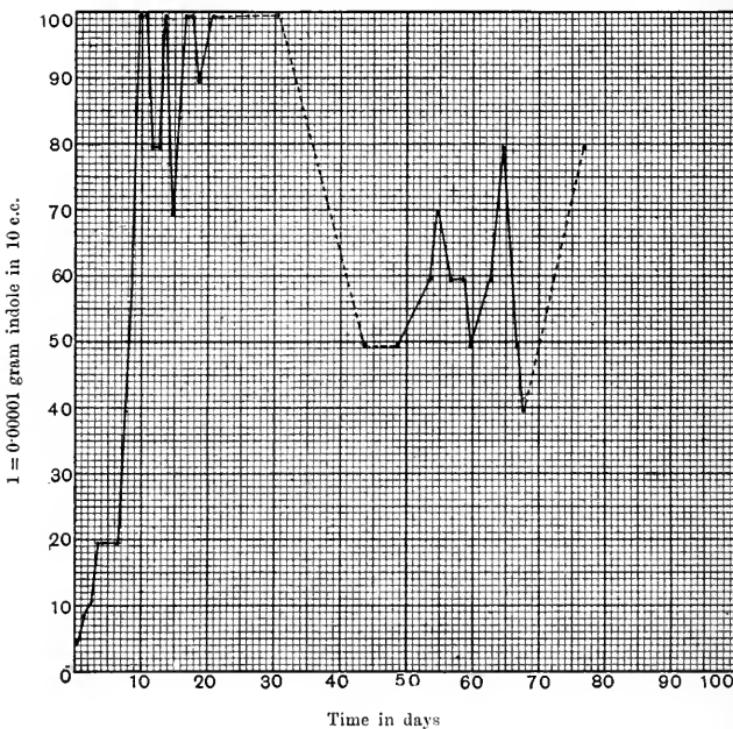


Chart I. *B. coli* Esch. a.

circumstance may influence the reactions, especially after two or three months when the quantity is much reduced. I do not think, however, that it would be advisable to proceed in any other way, as strong objections can be made against the two other procedures which suggest themselves, namely air-tight closing of the tubes, and the use of a flask culture, from which the quantity necessary for the test could be taken

daily. Air-tight closing would have the inconvenience that the reaction might proceed possibly in a different manner, when the free access of oxygen was excluded. If a flask culture was used, it would not be possible to obtain results of a uniform value; by abstracting from the culture 10 c.c. on, for instance, the 50th day, this amount would represent a far larger quantity of the original culture, which by then

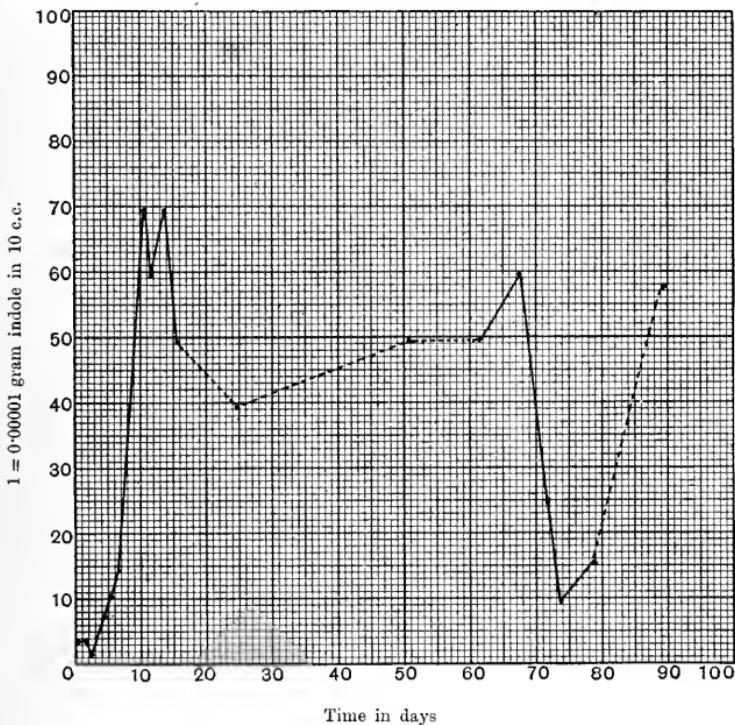


Chart II. *B. coli* (Pig faeces I).

would have become concentrated by evaporation. Consequently no comparison would be possible.

The advantage of the procedure adopted is, that each tube always contains the essential elements corresponding to the same quantity, 10 c.c., of the original peptone-water, notwithstanding the evaporation

of the water. The same amounts of the reagents were always used and, more especially, the same quantity of chloroform for extraction.

For the appreciation of the results it may be necessary to state that the comparative colorimetric scale in use consists of a series of tubes, the first one corresponding to a quantity 0·00001 gram indole to 10 c.c. of water and the following tubes to multiples of this, No. 100 consequently

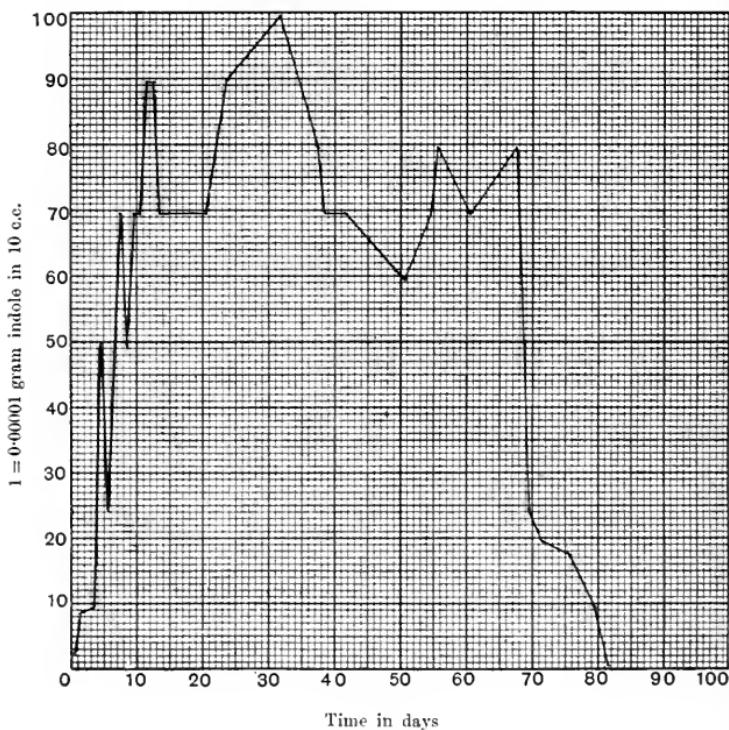


Chart III. *B. coli* (Pig faeces II).

to 0·001 gram in 10 c.c. For further detail the previous paper must be consulted.

The results obtained need no detailed discussion, as all particulars will become evident by a glance at the charts. All the strains examined were fairly strong indole producers; in all, with exception of *B. coli* (strain "Pig faeces I") represented in Chart II, a maximum of 100 or

just over, according to the scale, was obtained. The time, when the maximum was reached, differed considerably. The rise was in most cases gradual, although not quite regular and no remissions were seen before the maximum was reached. Afterwards, the curve lost its regularity and considerable oscillations occurred before a definite decrease began. The decrease was fully observed in two cases only (Charts III and V)

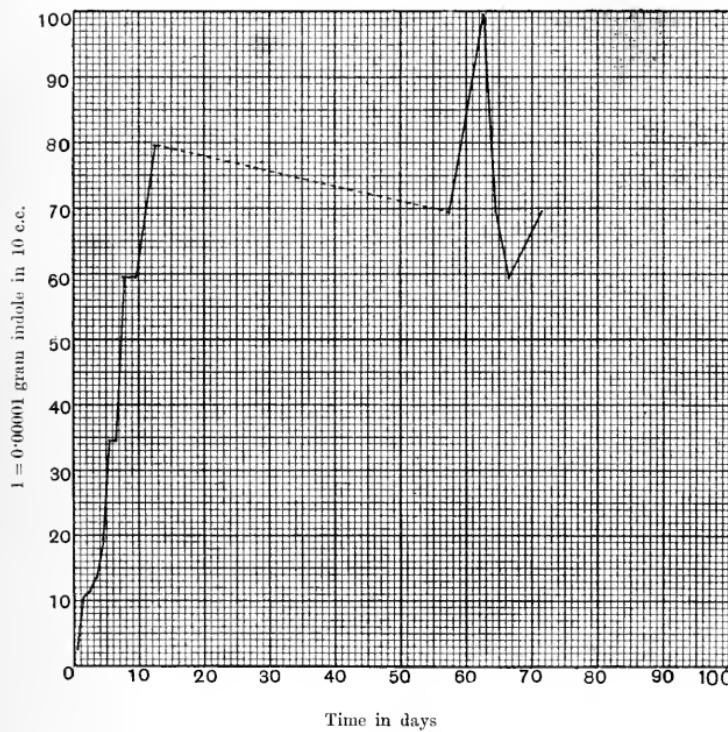


Chart IV. *B. coli* (Pig faeces III).

and in those the reaction disappeared between the 80th and 100th day.

The most interesting result is that indole was found present in the cultures, in considerable quantity, even when the tubes had been incubated for more than two months. Earlier authors, as Germano and Maurea (1893) and Marshall (1907) found a maximum of indole before

the end of the second week and did not follow the question up further.

So far, I cannot claim much value for the quantitative estimation of indole as a means of differentiating one strain of *B. coli* from another.

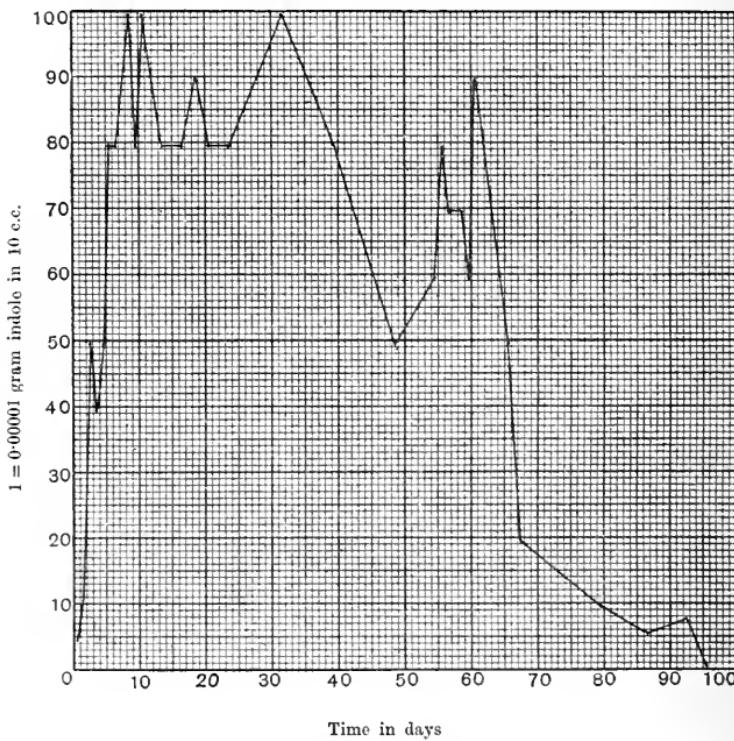


Chart V. *B. coli* Lab.

In a few cases it may be of use; in the strain represented in Chart II, for instance, the amounts of indole never approached those observed in the other strains. But in other cases the variations in cultures of the same strain were considerable. It is probable that the procedure has more interest in the study of the metabolism of bacteria.

In two of the strains a peculiar phenomenon was observed. When making the indole reaction in peptone-water cultures which had been

incubated for about eight days, a purple colour resulted instead of the ordinary red; by extraction with chloroform, only the red component passed into the extract, whilst the watery liquid took a blue colour, with the faintest possible trace of purple. Similar results were obtained when amyl-alcohol was used as the extracting liquid instead of chloroform.

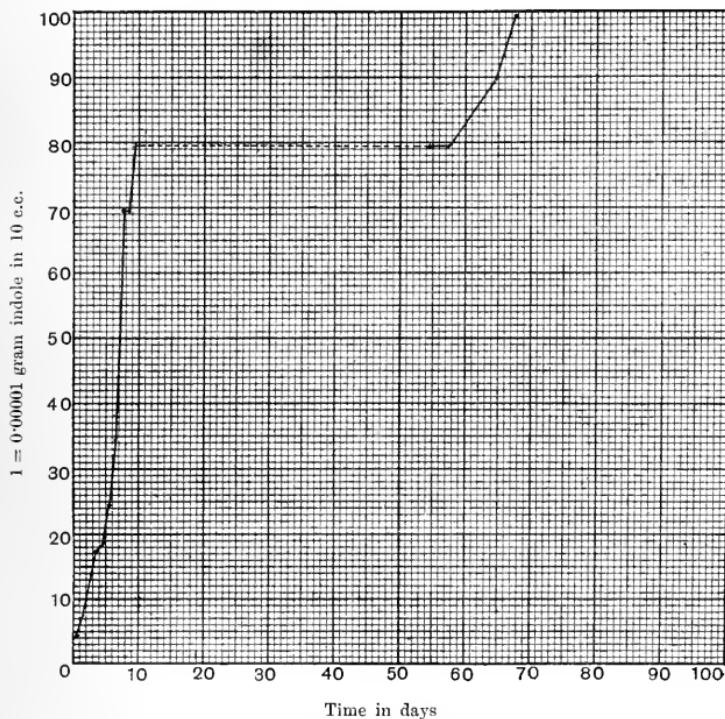


Chart VI. *B. coli* Esch. b.

The blue colour was first obtained in the course of the ordinary work with Ehrlich's reaction, executed in the manner described in my previous paper; afterwards a still more beautiful blue resulted by the use of a modified Steensma's (1906) reaction. The only difference between the two reactions, as I used them, was, that in Steensma's reaction a 0·5% sodium nitrite solution was substituted for the 1%

potassium persulphate in Ehrlich's, of the former only about ten drops being required.

Subsequently, similar reactions were observed with considerable frequency in the same two strains, but by no means constantly, nor always equally pronounced. It might even happen that several tubes, with identical quantities of peptone-water, inoculated at the same time and incubated for the same period gave quite different reactions. In one the colour after extraction might be quite blue, in another more purple, and in a third, almost red. All these coloured elements appeared, however, different from the ordinary red observed in the indole reaction, as even the red one could not be extracted by means of chloroform. The underlying causes of these differences are now being investigated.

The first idea, when a purple reaction was obtained, was, of course, that skatole might be present besides indole. That the blue colour was not due to skatole, became, however, evident when it failed to pass into chloroform and amyl-alcohol by extraction with these two substances, nor was the skatole reaction, with conc. sulphuric acid, according to the method of Sasaki (1910) obtained.

I was unable at that time to make further reactions, but Mr Lewis, to whom I demonstrated my results, soon found the same and similar phenomena in other cultures, in which he had already at an earlier date noticed a peculiar dark colour by Ehrlich's reaction, and undertook a further study of the substances concerned. A number of reactions were made, which Mr Lewis will communicate in the second part of this paper. We are now continuing these researches together and intend to publish the results at a later date.

Another interesting observation was made during my work with these bacteria. Ellinger and Gentzen (1904) found that the introduction of tryptophane into the coccus of rabbits gave rise to the excretion of a considerable amount of indican through the urine. The authors conclude that tryptophane represents a transitional stage in the formation of indole from proteins in the digestive tract.

This conclusion does not appear to be warranted by the experiments described; indole may well be formed from tryptophane when this substance happens to be present, but there is no proof that the process is a natural one. Still less justified of course would such a conclusion be concerning the *rôle* of tryptophane in the protein-decomposition by bacteria in general—an even more fundamental question. In order to throw some light on this question it seemed important to investigate

whether tryptophane could be demonstrated as a transitional product in cultures of indole-forming bacteria.

I therefore made the reaction for tryptophane with bromine water, as described by Abderhalden (1909), in peptone-water cultures of various strains, which were used in the other work mentioned in this paper. The reaction was made at different times, from a few hours to several days after the incubation of the cultures, both before my indole reaction had become apparent and when it was already quite strong. I never found the slightest indication of a positive result. That the reaction was not at fault, was proved by adding extremely small quantities of tryptophane to pure, uninoculated peptone-water and also to *B. coli*-cultures in peptone-water; a characteristic reaction was always obtained.

The result is therefore distinctly opposed to the conclusion drawn by Ellinger and Gentzen, but not to the result of their experiments. On the contrary, it is easy to demonstrate the formation of indole from tryptophane, by inoculating a tryptophane solution, with an indole-producing bacterium. I used a 0·5% tryptophane solution (with 0·5% NaCl) and obtained already on the following day a very strong indole reaction. The intensity of the indole reaction increased in the course of several days following and the tryptophane reaction, which was easily obtained even when the indole reaction had already become strong, disappeared in about a week.

Most of the work here detailed was executed at the Lister Institute, to the Director of which, Dr C. J. Martin, and to the Chief of the Bacteriological Department, Dr Ledingham, I feel greatly indebted.

## PART II.

By FREDERICK C. LEWIS.

In applying Ehrlich's indole reaction I observed that in many cultures the colour produced was exceedingly pronounced, especially in those cases in which the tubes had been left standing for some hours. In such cases the colour was decidedly purple. Upon extraction with chloroform, as suggested by Dr Seidelin, I was able to confirm what he had already observed, namely the production of a blue colour in addition to the red due to presence of indole.

This phenomenon was then further studied and other points of interest demonstrated.

At least three reactions may take place other than the production of the characteristic red colour due to the presence of indole.

Group 1. The production of a "double red" colour.

Group 2. The production of a purple or blue colour in the presence of indole.

Group 3. The production of a blue or somewhat blue purple colour *in the absence of indole*.

The technique followed was according to the modifications proposed by Seidelin (1911):

After the incubation of a suitable culture 5 c.c. of the paradigmethyl-amidobenzaldehyde reagent is added, followed by 5 c.c. of 1% potassium persulphate. The cultures are then left for a time and finally extracted with 5 c.c. of chloroform.

The length of time elapsing from the commencement of the reaction to the extraction with chloroform is a factor of great importance.

Group No. 1. (Production of the "double red" colour.)

An ordinary positive indole reaction is observed but, upon extraction with chloroform, not only is the chloroform coloured with the indole-red, but the supernatant fluid also remains red. These two red-coloured layers may now be separated and the upper stratum repeatedly washed with chloroform until no more colour can be extracted, i.e. until the chloroform remains colourless, whilst the upper stratum retains its red colour unchanged.

Group No. 2. (Purple or blue colour with a positive indole reaction.)

The reaction is similar to the above in its initial stages, but upon extraction with chloroform the indole-red is dissolved out leaving a purple upper stratum. This layer may now be separated and thoroughly washed with chloroform, the result being that when the last traces of indole-red are extracted the upper stratum is purple, with a more or less pronounced bluish tint, while the chloroform is as before perfectly colourless. The occurrence of this reaction is comparatively frequent.

If a series of tubes of peptone-salt solution be inoculated with a positive strain incubated for given periods and then tested daily, it will be observed that there is an increase, not only of the concentration of indole-red from day to day, but, also, of the purple colour.

At the same time, the differences which depend on the extraction being made shortly or a considerable time after the addition of the reagents, may be demonstrated. For example, in the following experiment, a series of tubes were inoculated with a positive strain and

incubated for periods of 24 hours, 48 hours etc., up to six days. At the end of 24 hours' incubation a culture was submitted to the ordinary technique without chloroform extraction and then divided into two equal parts *A* and *B*. Tube *A* was extracted with chloroform at the expiration of 30 minutes, and tube *B* was left standing for 24 hours before extracting. This process was repeated with each of the six cultures, after the periods of incubation previously mentioned.

The following facts were observed.

1. The intensity of both the red and purple colour was increased from day to day, and perfectly visible even on the first day.
2. In tube *A* the indole-red showed invariably a greater intensity than in tube *B*, whilst the inverse condition was observed with regard to the purple colour.

In passing, it may be noted that this purple colour is capable of destruction by the aid of suitable reducing agents and of being reformed by adequate oxidation, also, that it is not produced unless the reagents are added in relative excess of that required to produce the indole reaction.

Group No. 3. (Formation of a blue colour with negative indole reaction.)

In this reaction the final result is the formation of a distinct blue colour without the admixture of any, extractible or non-extractible, red. Primarily there may be a slight reddening, but this colouration soon disappears and gives place to a more or less intense greenish tint.

The initial red colour is not extractible by chloroform or amyl-alcohol and is consequently not due to the presence of indole. If the extraction by means of chloroform or amyl-alcohol is made when the change of colour to green has already taken place, a separation into two layers is observed as before, the chloroform or amyl-alcohol layer being colourless while the watery layer is blue of an intensity varying, not only with the period of incubation but also with the particular bacterial strain. Chloroform extraction gives the better result. The use of sodium nitrite solution in the place of potassium persulphate produces a similar reaction, but the final colour is a clear green instead of blue. Similar to the phenomena of purple colouration, a greater intensity of the reaction is obtained if the cultures are left for several hours prior to extraction, after the addition of the reagents. A number of different strains of *B. coli* have been isolated which give this reaction; it has also been found in certain strains of *B. enteritidis* Gaertner, *B. paratyphi* A, *B. lactis viscosus* and *B. prodigiosus*.

When the reaction is but slightly pronounced, a comparison should be made with a tube of sterile peptone-water of the same stock, after addition of the reagents and extraction in identically the same manner, as a slight change of colour may also be observed in this way.

I wish to take this opportunity of expressing my gratitude to Associate Professor Glynn for the kind interest with which he has followed my investigations.

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## THE SERUM REACTIONS (COMPLEMENT FIXATION) OF THE MENINGOCOCCUS AND THE GONOCOCCUS.

By J. A. ARKWRIGHT, M.D.

(*From the Bacteriological Department, Lister Institute.*)

THE present communication contains a record of further attempts to establish by serological methods some criterion for the differentiation of the *Meningococcus* from the *Gonococcus*.

According to the usually accepted views of the classification of the Gram-negative cocci, an organism which occurs on what may be called neutral ground, e.g., the human wrist-joint, and which resembles the Meningococeus in morphology and staining reactions and will not grow on artificial media except at a temperature above 22° C., must be regarded as a Meningococcus or a Gonocoecus or may possibly belong to the group of Gram-negative cocci called Pseudo-meningococci, amongst which should perhaps be included the *Micrococcus pharyngitidis flavus* III. of v. Lingelsheim.

The Pseudo-meningocoecus as described by Kutscher (1906), Lieberknecht (1908), Elsler and Huntoon (1909) and others, is an organism or group of organisms which occurs fairly frequently in the healthy human naso-pharynx and which resembles the Meningocoecus culturally. According to these writers it can, however, be distinguished with varying degrees of certainty from the true Meningocoecus by the agglutination test. My own observations (Arkwright, 1909) on the agglutination of different strains of Meningocoecus derived from the central nervous system, lead me to place little reliance on this method as a sure test to apply to an unknown strain from a doubtful source.

Dopter (1909 II.) described strains of Meningocoecus-like organisms occurring in the naso-pharynx, which were not agglutinated by a meningococcal serum, but which gave a complement-fixation reaction with the same serum. To these strains he gave the name of Parameningococcus. Recently the same writer (Dopter, 1911) has

described seven cases of sporadic meningitis from all of which he isolated the 'Parameningococcus.' Probably Dopter's strains of 'Parameningococcus' belong to the same category as the strains of Meningococcus which have been isolated by various workers (Eberle, Trautmann, Arkwright, etc.) from the meninges in cases of meningitis, but which have not been found to react with meningococcal sera prepared with other strains of Meningococcus.

It seems, however, reasonable to expect that it would be much easier to distinguish by serological methods the Gonococcus from the Meningococcus than the latter from an organism resembling it so closely as the Pseudo-meningococcus.

In spite of the usually fairly obvious cultural differences between the Gonococcus and the Meningococcus and the sharp line that is to be drawn between the sources and pathological associations of these two organisms, experience does not bear out the anticipation of wide divergence as regards serum reactions.

*Brief review of the methods advocated for the differentiation  
of the Meningococcus from the Gonococcus.*

*I. Cultural and biochemical tests.*

(1) Meningococci grow on media with a much wider range of alkalinity than do Gonococci, and after the first few subcultures Meningococci will almost invariably grow well on ordinary neutral agar. This is not the case with the Gonococcus which requires either serum-agar or a specially prepared agar distinctly alkaline to litmus as recommended by Thalmann (1900, 1902) or a medium prepared with phosphate of soda as used by Blair Martin (1910). It can, however, easily be shown that different strains of Gonococcus prefer different degrees of alkalinity, and many observers have noticed that some strains of Gonococcus quickly become accustomed to ordinary neutral agar.

(2) On serum-agar, or agar of a suitable reaction, the colonies after 24-hours' growth in the case of the Gonococcus are always discrete and very small or pin point, whereas the colonies of the Meningococcus on the same medium are frequently confluent or, if few and discrete, are of much larger size.

These cultural characters on solid media appear to the writer to be the most constant features which differentiate cultures of the Gonococcus from those of the Meningococcus—an opinion also expressed by Wollstein (1907).

(3) Growth on carbohydrate media has been widely used and found of considerable value as a means of differentiation. Most observers, e.g. v. Lingelsheim, Elsler and Huntoon, Blair Martin and others have laid stress on the inability of the Gonococcus to ferment maltose, but I have not found this to be a constant distinguishing feature. Some strains of Gonococcus produce a distinct acidity when grown on serum-peptone-water or serum-agar to which pure maltose has been added (Arkwright, 1907, 1909; Wollstein, 1907; Gurd, 1908), and I have met with undoubted strains of Meningococcus from the cerebro-spinal fluid of cases of meningitis which did not ferment maltose. In considering these sugar reactions it must be borne in mind that the growth of Gonococcus on culture media neutral to litmus is usually very feeble and the production of acid from glucose is also less than in the case of the Meningococcus.

## II. Serological tests.

I. *Agglutination.* Though many writers, v. Lingelsheim (1906), Kutseher (1906), Krumbein and Schatiloff (1908) and others have described very uniform results obtained with meningococcal serum and different strains of Meningococcus, this has not been by any means the universal experience of workers.

The objection to placing reliance on agglutination as a means of recognising the members of the different groups is based on two sets of facts: (1) the want of uniformity obtained with different members of the same group, e.g., the Meningococcus, when a meningococcal serum is used (Trautmann and Fromme, 1908; Eberle, 1908; Ditthorn and Gildermeister, 1907; Lieberknecht, 1908; Elsler and Huntoon, 1909; Arkwright, 1909), and (2) the fact that some strains of Meningococcus will agglutinate with a gonococcal serum and *vice versa* (Vannod, 1906; Dopter and Koch, 1908 vii.; Elsler and Huntoon, 1909; Wollstein, 1907). In the last case the experiments are further complicated by the difficulty of obtaining good uniform emulsions of the Gonococcus.

I found that, when working with a monovalent meningococcal serum, the number of strains agglutinated was very limited, and even when a polyvalent serum of a titre of 1-1000 made by injecting twelve different strains of Meningococcus was employed, strains of Meningococcus were easily found which were not agglutinated more highly by the specific serum than by normal serum (Arkwright, 1909).

Possibly the greater uniformity obtained by some observers has been due to the use of strains all of which occurred in the same epidemic

and to the employment of polyvalent sera made with many such strains.

It has been claimed by Dopter and Koch (1908 VII.) that by the use of the method of absorption of agglutinins a specific agglutination reaction can be demonstrated, even when a given serum agglutinates both the Meningococcus and the Gonococcus equally before absorption.

Since, however, a meningococcal serum may agglutinate some strains of Gonococcus but affect only a limited number of strains of Meningococcus, the method of absorption can only have a very restricted application.

Moreover absorption experiments (Arkwright, 1909) with different strains of Meningococcus and a meningococcal serum gave a differentiation between the strains of Meningococcus used similar to that obtained by Dopter and Koch between the Meningococcus and the Gonococcus. Torrey (1907) obtained results of the same kind in his studies on the agglutination of the Gonococcus.

II. By means of the Opsonic index Houston and Rankin (1907) claimed to be able to distinguish epidemic from sporadic strains of Meningococcus. This method according to their results would be quite useless for distinguishing the Gonococcus from the Meningococcus. Wollstein (1907) was unable to distinguish by opsonic experiments the Gonococcus from the Meningococcus.

III. The Precipitin reaction has been advocated by Dopter and Koch (1908 x.) and Dopter (1909 I.) as a means of differentiation in this group of organisms, but the experiments recorded by them were too few in number to justify the deduction of definite conclusions. Dopter carried the differentiation further by absorbing the precipitins.

IV. Complement fixation when applied for the same purpose has given very varying results. Vannod (1906) and Krumbein and Schatiloff (1908) consider this reaction specific for the Meningococcus and for the Gonococcus with their respective sera. Krumbein and Schatiloff used a polyvalent meningococcal serum and also gonococcal serum. Watabiki (1910), though not obtaining such distinct results with the two groups of cocci, maintained that the Gonococcus could be differentiated from other cocci by this reaction. Arkwright (1909) found this method in no way superior to agglutination as a means of distinguishing the Meningococcus from the Gonococcus. Wollstein (1907) working with monovalent sera found no distinction between the Meningococcus and the Gonococcus. Colombo (1911) has recently published a series of observations on complement fixation with these two organisms and

has found no specific difference between them. He used almost entirely polyvalent sera.

The experience of various observers as regards agglutination suggested that the use of polyvalent sera for other serum reactions in this group of organisms might be delusory. Meningococci and Gonococci appear to fall, as regards agglutination, into subgroups the members of which react among themselves, but not with the members of other subgroups of the same organism (Torrey, 1907; Arkwright, 1909). Unless, therefore, the serum used were obtained by injecting all the subgroups, it might fail to produce the reaction with the cocci which were members of the remaining subgroups and which had not been injected. A polyvalent serum might give very uniform reactions with six strains but not with a seventh or eighth. On the other hand, although a polyvalent meningococcal serum gives a reaction with some strains of Gonococcus as well as with some strains of Meningococcus, it is possible that a monovalent meningococcal serum might be obtained which had no affinities with any strain of Gonococcus. If such a monovalent meningococcal serum gave uniformly positive results with all strains of Meningococcus and negative results with all the strains of Gonococcus which were available, the result would be significant and the use of such a serum for the classification of new strains might be of value. Unless, however, a serum with such strictly specific activity can be obtained, serological tests are not of much value for making a final and conclusive diagnosis of a given strain of a meningococcus-like organism.

A polyvalent serum is very unlikely to fulfil these conditions and consequently a series of monovalent meningococcal and gonococcal sera were prepared with the object of testing the specificity of the complement-fixation reaction with monovalent sera.

#### *Preparation of immune sera.*

For my experiments monovalent sera were prepared by injecting rabbits intravenously with emulsions of cocci in increasing doses. Heated cocci were used first, but for the later injections living cultures were employed. Considerable difficulty was experienced in obtaining sera of sufficient strength, as the rabbits frequently died after the later doses, especially when the Gonococci were being used.

The strains of Meningococcus and Gonococcus used for injection were grown on horse serum agar.

Eventually three monovalent meningococcal and five monovalent gonococcal sera were obtained which gave fairly well marked complement fixation with the homologous cocci or extracts.

Experiments were also made with one polyvalent serum and one monovalent serum obtained from the horse.

#### *Strains used.*

The strains of Meningococcus and Gonococcus used in these experiments were the following:

##### *Meningococci :*

- M. XII* Isolated from the meninges of a sporadic case of menigitis.  
*M. 119* Isolated post-mortem from the spinal cord of a case of acute epidemic meningitis.  
*M. 135* Isolated from the cerebro-spinal fluid of a sporadic case of meningitis.  
*M. 141* From the cerebro-spinal fluid of a sporadic case of meningitis.  
*M. 162* From the cerebro-spinal fluid of a sporadic case of meningitis.  
*M. 164* From the cerebro-spinal fluid of a very acute sporadic case of meningitis.  
*M. 165* From the cerebro-spinal fluid of an adult case of meningitis occurring in an epidemic area.

##### *Gonococci :*

- G. 1* Isolated from the vaginal discharge of a child suffering from vulvo-vaginitis.  
*G. 2* From a case of acute gonorrhoea in an adult male.  
*G. 3* From another case of acute gonorrhoea in an adult male.  
*G. 4* From a case of acute gonorrhoea in an adult male.  
*G. 6* From a case of vulvo-vaginitis in a child.

#### *Preparation of the antigen extract.*

Extracts of the cocci were used as "antigen" for the complement-fixation reaction and various methods of extraction were tried. The following method was found to be the best of those experimented with:

An emulsion of the growth on ascitic agar in a Roux bottle was made with 10 c.c. of salt solution. This was centrifuged and the deposit made up to its original volume with salt solution. After adding a few drops of chloroform and shaking, the emulsion was left at room temperature for three or four days. The extract was then centrifuged before use. It was found that if the deposit from the last centrifuging was again made up to the original volume with salt solution and left for a further period of two to three days, a second extract as good as the first could be obtained, and by again repeating the same process, a third

and even a fourth extract could sometimes be obtained of almost undiminished value for complement-fixation experiments.

The extract was diluted eight or in some cases sixteen times before use and the specific meningococcal or gonococcal serum was diluted eight times. The largest dose of serum or extract diluted as above was 0·5 c.c. and a tube containing a double dose of serum and another with a double dose of extract were always put up as controls. Some experiments were made in which falling doses of extract were used with a constant dose of specific serum, and other experiments in which the dose of extract was constant but the doses of serum decreased in the successive tubes. The results yielded by the two methods were on the whole alike.

#### *Haemolytic system.*

The haemolytic system used consisted of sheep's corpuscles, rabbit-v.-sheep serum, and guinea-pig complement. The haemolytic serum was titrated each day with the complement and a double haemolytic dose of serum was used with 0·5 c.c. of a 1-10 dilution of fresh guinea-pig's serum.

No results are recorded unless the control tubes gave complete haemolysis.

The controls have been omitted from the tables for the sake of brevity.

The signs used in the tables indicate the amount of haemolysis which took place. Thus + + + = complete haemolysis; + + = partial haemolysis; + = slight haemolysis; and - = no haemolysis.

#### *Experiment I.*

In Experiment I the following sera and extracts of cocci were employed :

<i>M.M.H.S. (XII)</i> =	Monovalent meningococcal horse serum obtained by immunisation with <i>Meningococcus XII</i> .
<i>P.M.H.S.</i> =	Polyvalent meningococcal horse serum obtained by immunisation with 20 strains of <i>Meningococcus</i> .
<i>M.E. 141</i> =	Meningococcal extract prepared from <i>Meningococcus 141</i> .
<i>M.E. 162</i> =	Meningococcal extract prepared from <i>Meningococcus 162</i> .
<i>G.E. 1</i> =	Gonococcal extract prepared from <i>Gonococcus 1</i> .

The meningococcal extracts are heterologous to both sera.

The dose of extract was kept constant and used with falling doses of serum.

The results of Experiment I are shown in Table I:

TABLE I.

Sera <i>M.M.H.S. (XII)</i>	Dose	Extracts		
		<i>M. 141</i>	<i>M. 162</i>	<i>G. I</i>
0·5	0·5	—	+	—
0·25	0·5	—	++	—
0·125	0·5	+	+++	+
0·06	0·5	++	+++	+
0·03	0·5	+++	+++	...
<i>P.M.H.S.</i>				
0·5	0·5	—	—	—
0·25	0·5	—	—	—
0·125	0·5	—	—	—
0·06	0·5	+	+	+
0·03	0·5	++	++	+

It will be seen that the monovalent meningocoecal serum produced fixation of complement to an equal degree in the presence of the Gonococcus extract and one of the heterologous Meningococcus extracts, but in the presence of the other Meningococcus extract complement was bound only to a very slight extent. The polyvalent meningocoecal serum on the other hand produces fixation of complement equally in the presence of all three extracts.

#### *Experiment II.*

In Exp. II the same two horse sera and meningocoecal extracts were used, but a third meningococcal extract was also tested with these sera.

In this experiment the dose of serum was kept constant and falling doses of extract were employed.

The results obtained in Exp. II are shown in Table II. Tested in this way the difference between the reactions with *M.E. 141* and *162* were still well marked. The monovalent serum caused marked complement-fixation in the presence of only one of the extracts (*M.E. 141*) but in the presence of *M.E. 119* or *M.E. 162* it produced very slight fixation.

When the polyvalent meningocoecal serum was employed, rather different results were obtained. *M.E. 119* still gave a completely negative reaction, whereas a difference was shown between *M.E. 141* and *M.E. 162*, which did not appear in Exp. I when falling doses of serum were used.

TABLE II.

Sera <i>M.M.H.S. (XII)</i>	Dose	Extracts		
		<i>M. 119</i>	<i>M. 141</i>	<i>M. 162</i>
0·5	0·5	+++	-	++
0·5	0·25	+++	-	+++
0·5	0·125	+++	-	+++
0·5	0·06	+++	+	+++
0·5	0·03	+++	++	+++

<i>P.M.H.S.</i>	Dose	Extracts		
		<i>M. 119</i>	<i>M. 141</i>	<i>M. 162</i>
0·5	0·5	+++	-	-
0·5	0·25	+++	-	+
0·5	0·125	+++	+	+++
0·5	0·06	+++	+	+++
0·5	0·03	+++	++	++

## Experiment III.

In this experiment two monovalent rabbit sera were used and extracts of six strains of Meningococcus and of one strain of Gonococcus.

*M.M.R.S. 141* = Monovalent meningococcal rabbit serum prepared with *M. 141*.

*M.G.R.S. 1* = Monovalent gonococcal rabbit serum prepared with *G. 1*.

*M.E. 119* = Extract of *Meningococcus 119*.

*G.E. 1* = Extract of *Gonococcus 1*.

Etc.

The doses of serum were kept constant and falling doses of extract were used as in Exp. II.

TABLE III.

Sera <i>M.M.R.S. 141</i>	Dose	Extracts						<i>G. 1</i>
		<i>M. 119</i>	<i>M. 135</i>	<i>M. 141</i>	<i>M. 162</i>	<i>M. 164</i>	<i>M. 165</i>	
0·5	0·5	+	-	-	-	-	-	-
0·5	0·25	++	+	-	++	+	+	-
0·5	0·125	+++	++	+	++	++	++	+
0·5	0·06	+++	+++	++	+++	++	++	+++
0·5	0·03	+++	+++	+++	+++	+++	+++	+++

<i>M.G.R.S. 1</i>	Dose	Extracts						<i>G. 1</i>
		<i>M. 119</i>	<i>M. 135</i>	<i>M. 141</i>	<i>M. 162</i>	<i>M. 164</i>	<i>M. 165</i>	
0·5	0·5	+	-	-	+	-	-	-
0·5	0·25	++	-	-	++	++	+	-
0·5	0·125	++	++	++	++	++	++	+
0·5	0·06	++	++	++	++	++	++	++
0·5	0·03	++	++	++	++	++	++	++

The results of Exp. III recorded in Table III show that the degree of complement fixation produced by the monovalent gonococcal serum and by the monovalent meningococcal serum in the presence of the same gonococcal and meningococcal extracts was almost the same. Also the meningococcal serum caused fixation of complement to the same extent whether the homologous meningococcal extract or a gonococcal extract was used. On the other hand in the presence of each of five heterologous meningococcal extracts this meningococcal serum bound complement less than when in association with the gonococcal extract.

#### *Experiment IV.*

Sera prepared with the same two cocci were used in Exp. IV as in Exp. III and in addition a gonococeal serum was employed.

<i>M.M.R.S. III</i>	=	Monovalent meningococcal rabbit serum prepared with <i>M. III</i> .
<i>M.G.R.S. I</i>	=	Monovalent gonococcal rabbit serum prepared with <i>G. I</i> .
<i>M.G.R.S. 2</i>	=	Monovalent gonococcal rabbit serum prepared with <i>G. 2</i> .
<i>M.E. 135</i>	=	Extract of <i>M. 135</i> .
<i>M.E. III</i>	=	Extract of <i>M. III</i> .
<i>G.E. I</i>	=	Extract of <i>G. I</i> .

The dose of extract was kept constant in this experiment and falling doses of serum were used.

TABLE IV.

Sera	Dose	Extracts		
		<i>M. 135</i>	<i>M. III</i>	<i>G. I</i>
<i>M.M.R.S. III</i>	0·5	0·5	—	—
	0·25	0·5	—	—
	0·125	0·5	+	—
	0·06	0·5	++	+
	0·03	0·5	+++	+++
<i>M.G.R.S. I</i>	0·5	0·5	—	—
	0·25	0·5	—	—
	0·125	0·5	+	—
	0·06	0·5	++	—
	0·03	0·5	+++	+
<i>M.G.R.S. 2</i>	0·5	0·5	—	—
	0·25	0·5	+	—
	0·125	0·5	++	—
	0·06	0·5	++	—
	0·03	0·5	+++	—

The results obtained in Exp. IV and shown in Table IV appear at first sight to suggest that a strain of Gonococcus can be distinguished from a meningococcal strain by means of a gonococcal serum used in falling doses, but that this is not a constant result is shown by Exps. V and VI. In Table IV is again shown the very uniform degree of fixation of complement obtained with a monovalent meningococcal serum, and meningococcal and gonococcal extracts.

### *Experiment V.*

Three sera were tested in this experiment and three extracts of cocci. Falling doses of serum and constant doses of extract were used.

- |                       |   |
|-----------------------|---|
| <i>M.M.R.S. 162</i> = | Monovalent meningococcal rabbit serum prepared with <i>M. 162</i> . |
| <i>M.G.R.S. 3</i> =   | Monovalent gonococcal rabbit serum prepared with <i>G. 3</i> .      |
| <i>M.G.R.S. 4</i> =   | Monovalent gonococcal rabbit serum prepared with <i>G. 4</i> .      |

TABLE V.

Sera	Dose	Extracts		
		<i>M. 135</i>	<i>M. 162</i>	<i>G. 2</i>
<i>M.M.R.S. 162</i>	0·5	+	-	
	0·25	-	-	-
	0·125	+	-	-
	0·06	++	+	-
	0·03	+++	++	-
<i>M.G.R.S. 3</i>	0·5	++	++	-
	0·25	++	+++	-
	0·125	+++	+++	-
	0·06	+++	+++	-
	0·03	+++	+++	+
<i>M.G.R.S. 4</i>	0·5	-	-	-
	0·25	-	-	-
	0·125	++	++	+
	0·06	++	++	++
	0·03	++	+++	+++

Table V shows the results of complement-fixation obtained in Exp. V. The most noticeable fact is that the gonococcal extract in the presence of either meningococcal (*162*) or gonococcal (*3*) serum produced a greater degree of complement-fixation than either of the meningococcal extracts. When, however, Gonococcus serum (*4*) was used, the three coccal extracts (*M. 135*, *M. 162* and *G. 2*) all produced nearly the same degree of fixation.

*Experiment VI.*

In Experiment VI two meningococcal sera were used, three meningoeoccal extracts and one gonococcal extract. Falling doses of serum and constant doses of extract were again employed.

TABLE VI.

Sera <i>M.M.R.S. 141</i>	Dose	Extracts			
		<i>M. 141</i>	<i>M. 162</i>	<i>M. 164</i>	<i>G. 6</i>
0·5	0·5	—	+	+	—
0·25	0·5	+	+	++	..
0·125	0·5	+	++	++	—
0·06	0·5	++	++	++	+
0·03	0·5	+++	+++	+++	++
<i>M.M.R.S. 162</i>					
0·5	0·5	—	—	+	—
0·25	0·5	—	—	—	—
0·125	0·5	—	—	—	—
0·06	0·5	—	—	+	—
0·03	0·5	+	—	+	—

Table VI shows the results of Experiment VI. Here again the gonococcal extract was more efficient for complement-fixation than the meningoeoccal extracts, even although the sera used were monovalent sera. This superiority is especially well shown in the upper part of the table where the results of fixation with a rather weak serum (*M.M.R.S. 141*) are recorded.

These experiments appear to show that though a monovalent serum produces generally a more marked complement-fixing reaction with its homologous strain of coccus than with heterologous strains, nevertheless such a serum has not necessarily any greater affinity for the other strains of the same group (Meningococcus or Gonococcus) than for strains of the other group.

It was, however, noticed (and this appears in the tables) that extracts prepared from strains of Gonococcus were on the whole more efficient for producing complement-fixation with a meningococcal or gonococcal serum than extracts made from strains of Meningococcus. Colombo (1911) arrived at a similar conclusion, both as regards the non-specificity of the complement-fixation reaction and the greater effect produced by gonococcal extract.

## SUMMARY AND DISCUSSION.

The results which were obtained in the foregoing six experiments and are detailed in Tables I to VI may be summarised as follows.

*Statement of complement-fixation reactions combined from all the Tables\*.*

Reaction of <i>M.M.H.S.</i> (XII) was	... Positive with <i>E.M. 141</i> and <i>E.G. 1</i> . Very slight with <i>E.M. 162</i> . Negative with <i>E.M. 119</i> and <i>E.M. 162</i> .
Reaction of <i>P.M.H.S.</i> (20 strains) was	Positive with <i>E.M. 141</i> , <i>E.M. 162</i> and <i>E.G. 1</i> . Negative with <i>E.M. 119</i> .
Reaction of <i>M.M.R.S. 141</i> was	... Positive with <i>E.M. 135</i> , <i>E.M. 141</i> , <i>E.M. 162</i> , <i>E.M. 164</i> , <i>E.M. 165</i> , <i>E.G. 1</i> and <i>E.G. 6</i> . Very slight with <i>E.M. 162</i> and <i>E.M. 164</i> .
Reaction of <i>M.M.R.S. 162</i> was	... Positive with <i>E.M. 135</i> , <i>E.M. 162</i> , <i>E.M. 164</i> , <i>E.G. 2</i> and <i>E.G. 6</i> .
Reaction of <i>M.G.R.S. 1</i> was	... Positive with <i>E.M. 135</i> , <i>E.M. 141</i> , <i>E.M. 164</i> , <i>E.M. 165</i> and <i>E.G. 1</i> . Negative with <i>E.M. 119</i> and <i>E.M. 162</i> .
Reaction of <i>M.G.R.S. 2</i> was	... Positive with <i>E.M. 135</i> and <i>E.G. 1</i> .
Reaction of <i>M.G.R.S. 3</i> was	... Positive with <i>E.G. 2</i> . Negative with <i>E.M. 135</i> and <i>E.M. 162</i> .
Reaction of <i>M.G.R.S. 4</i> was	... Positive with <i>E.M. 135</i> , <i>E.M. 162</i> and <i>E.G. 2</i> .

\* In some cases slightly different results were obtained on different occasions.

It is seen then that any attempt to classify these two groups of cocci by means of complement-binding reactions would arrange them into more or less well marked sub-groups, some of which would contain both meningococcal and gonococcal strains, and some perhaps strains from only one of these groups.

It will be noticed that extracts of three strains of Meningococcus (*119*, *162* and *164*) showed an especial tendency to give negative or feeble complement-fixation reactions with heterologous meningococcal sera, and two of these strains (*119* and *162*) also gave negative reactions with some of the gonococcal sera. Though the complement-fixation reactions of the strains used in this research were not fully worked out on account of the difficulty in obtaining satisfactory sera, nevertheless the following classification appears to be indicated having regard to complement-fixation alone.

- Sub-group I. *M. (XII)*, *M. 141*, *M. 165*, *G. 1*, *G. 3*.
- II. *M. 135*, *G. 2*.
- III. *M. 162*, *M. 164*, *G. 4*, *G. 6*.
- IV. *M. 119*.

Sub-group II has affinities for sub-groups I and III, but there is little, if any, affinity shown between I and III directly. *M. 119* shows slight affinity for *M. 141*, but for no other strains.

The sub-groups are not clearly defined, but overlap and are connected with each other in various directions. For instance *G. 1*, *M. 135* and *G. 2* all appear to have common receptors, and *M. 135* and *G. 2* also have receptors in common with *G. 4* and *M. 162*, but *G. 1* shows no affinity for these two latter strains.

The explanation of these facts is not quite simple but they may be explained by assuming, (1) that several group antigens occur which are common to the Meningococcus and the Gonococcus, but only some of which are present in any given strain of coccus, and (2) that specific antigens which are peculiar to the Meningococcus on the one hand or to the Gonococcus on the other hand do occur, but are often absent in the case of any given strain.

The second assumption is perhaps unnecessary, and the first is almost equivalent to affirming the occurrence of special antigens peculiar to certain sub-groups which contain strains of both Meningococcus and Gonococcus.

The evidence, then, from complement-fixation experiments as also from other serum tests as far as they are of any value, appears to point to a closer relationship between some strains of Gonococcus and some strains of Meningococcus than between different sub-groups of Meningococcus. In fact rather the unity of these two groups than any essential difference between them, is suggested by these facts.

These considerations lend further support to the view that the most constant bacteriological characters available for differentiating the Meningococcus from the Gonococcus are the cultural characters seen when the organisms are grown on agar of different degrees of alkalinity.

#### CONCLUSIONS.

(1) Meningococcal sera produce complement-fixation as readily with some gonococcal extracts as with extracts of some strains of Meningococci; whereas no reaction is obtained with some heterologous meningococcal extracts.

(2) A monovalent serum usually reacts better with an extract of its homologous coccus than with extracts of other strains of Meningococcus or Gonococcus, but a gonococcal extract sometimes gives a

better reaction with a meningococcal serum than the homologous extract does.

(3) Gonococcal sera and extracts are on the whole more potent than those prepared from Meningococci as regards complement-fixation.

(4) No satisfactory distinction between Meningococci and Gonococci can be demonstrated by means of complement-fixation tests.

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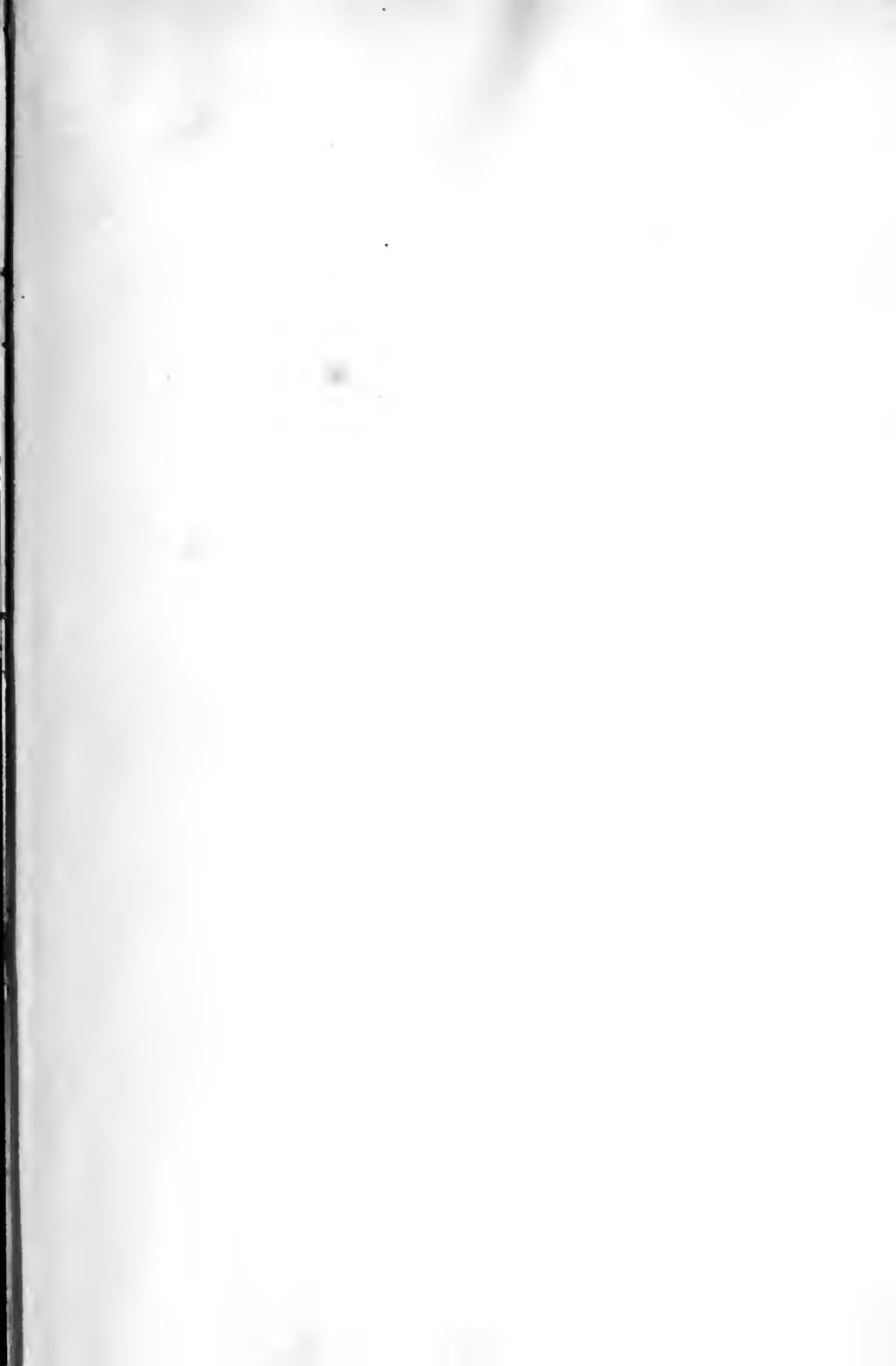
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